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(54) ANTI-HUMAN VEGF RECEPTOR F1t-1 MONOCLONAL ANTIBODY

(57) The present invention provides a monoclonal antibody which immunologically reacts with human VEGF receptor Flt-1 and cells in which human VEGF receptor Flt-1 is expressed on the cell surface and a monoclonal antibody which inhibits binding of human VEGF to human VEGF receptor Flt-1. It also provides a means for the diagnosis or treatment of diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity, psoriasis, and the like.

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Description

TECHNICAL FIELD

This invention relates to a monoclonal antibody capable of specifically binding to human VEGF receptor Flt-1 which is useful for the diagnosis or treatment of diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity and psoriasis; a hybridoma capable of producing the antibody; a method for immunologically detecting human VEGF receptor Flt-1 using the monoclonal antibody; and a diagnostic method and a therapeutic method for diseases, such as solid tumor, rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity, psoriasis and the like, using the monoclonal antibody.

BACKGROUND ART

Angiogenesis plays an important role in the individual development and construction of tissues in vertebrates, is directly involved in the formation of the corpus luteum during the sexual cycle, transient proliferation of the uterine endometrium and formation of the placenta in mature individuals (females). With regard to pathological states, angiogenesis is involved in the proliferation or metastasis of solid tumors and formation or acceleration of morbidity in diabetic retinopathy and rheumatoid arthritis (*J. Biol. Chem.*, 267, 10931 (1992)). Angiogenesis occurs by the secretion of an angiogenesis factor and involves the process of a tube formation and producing a new blood vessel. During this process, the basement membrane and interstitum are destroyed by a protease secreted from endothelial cells of an existing blood vessel around the secreted angiogenesis factor, followed by subsequent wandering and proliferation of vascular endothelial cells (*J. Biol. Chem.*, 267, 10931 (1992)). Factors which induce angiogenesis include vascular permeability factor (hereinafter "VPF") and vascular endothelial growth factor (hereinafter "VEGF") (hereinafter "VPF/VEGF"). These factors are considered the most important factors in pathological and non-pathological angiogenesis (*Advances in Cancer Research*, 67, 281 (1995)). VPF/VEGF is a protein having a molecular weight of about 40,000 constituted by homodimers, which had been reported to be independent molecules as vascular permeability factor (VPF) in 1983 (*Science*, 219, 983 (1983)) and as vascular endothelial growth factor (VEGF) in 1989 (*Biochem. Biophys. Res. Comm.*, 161, 851 (1989)), but it has been revealed as the results of cDNA cloning that they are the same substance (*Science*, 246, 1306 (1989); *Science* 246, 1309 (1989)) (hereinafter, the term "VPF/VEGF" is recited as "VEGF"). Beyond the activity of VEGF upon vascular endothelial cells described above, VEGF has also been shown to have a growth enhancing activity (*Biochem. Biophys. Res. Comm.*, 161, 851 (1989)), a migration enhancing activity (*J. Immunology*, 152, 4149 (1994)), a metalloprotease secretion enhancing activity (*J. Cell Physiol.*, 153, 557 (1992)), a urokinase and tPA secretion enhancing activity (*Biochem. Biophys. Res. Comm.*, 181, 902 (1991)), and the like. Furthermore, VEGF has been shown to have an angiogenesis enhancing activity (*Circulation*, 92 suppl II, 365 (1995)), a vascular permeability enhancing activity (*Science*, 219, 983 (1983)), and the like as its *in vivo* activities. It has been reported that VEGF is a growth factor having extremely high specificity for vascular endothelial cells (*Biochem. Biophys. Res. Comm.*, 161, 851 (1989)) and that four proteins having different molecular weight are present due to alternative splicing of mRNA (*J. Biol. Chem.*, 267, 26031 (1991)).

Among diseases accompanied by angiogenesis, it has been reported that VEGF plays an important role in the proliferation or metastasis of solid tumors and formation of morbid states of diabetic retinopathy and rheumatoid arthritis. With regard to solid tumors, production of VEGF in a number of human tumor tissues has been reported, such as in renal carcinoma (*Cancer Research*, 54, 4233 (1994)), breast cancer (*Human Pathology*, 26, 86 (1995)), brain tumor (*J. Clinical Investigation*, 91, 153 (1993)), gastrointestinal cancer (*Cancer Research*, 53, 4727 (1993)), ovarian cancer (*Cancer Research*, 54, 276 (1994)), and the like. Also, results of a study on the correlation between VEGF expression quantity in tumors and survival ratio of patients in patients with breast cancer have revealed that tumor angiogenesis is more active in tumors expressing high levels of VEGF than low VEGF expression tumors and that the survival ratio is lower in breast cancer patients having high VEGF expression tumors than breast cancer patients having low VEGF expression tumors (*Japanese J. Cancer Research*, 85, 1045 (1994)). It has been reported also that an anti-VEGF monoclonal antibody inhibited tumor growth in a xenograft model test system in which a human tumor was transferred into nude mice by subcutaneous transplantation (*Nature*, 362, 841 (1993)). Also, it has been reported that, in a metastatic cancer model of a human tumor in nude mice, an anti-VEGF monoclonal antibody inhibited metastasis of the tumor (*Cancer Research*, 56, 921 (1996)). Additionally, since a high concentration of VEGF was detected in human carcinomatous pleural perfusions and ascites, the possibility that VEGF is a major factor involved in the retention of pleural perfusions and ascites has been suggested (*Biochimica et Biophysica Acta*, 1221, 211 (1994)).

In diabetic retinopathy, abnormal angiogenesis causes retinal detachment and hemorrhage of the vitreous body, resulting in blindness, and it has been reported that angiogenesis in diabetic retinopathy and the expression level of VEGF in the patient's eye balls are positively correlative (*New England J. Medicine*, 1331, 1480 (1994)). Also, it has

been reported that angiogenesis in a monkey retinopathy model is inhibited when the VEGF activity is inhibited by the intraocular administration of an anti-VEGF n utralizing monoclonal antibody (*Arch. Ophthalmol.*, 114, 66 (1996)).

Progress in the morbid states of rheumatoid arthritis (destruction of bone and cartilage) is accompanied by angiogenesis, and it has been reported that a high concentration of VEGF is contained in the synovial fluid of pati nts with rheumatoid arthritis and that macrophages in joints of patients with rheumatoid produce VEGF rheumatoid arthritis (*Journal of Immunology*, 152, 4149 (1994); *J. Experimental Medicine*, 180, 341 (1994)).

VEGF receptors have been reported. These include fms-like tyrosine kinase (referred to as "Flt-1" hereinafter) (*Oncogene*, 5, 519 (1990); *Science*, 255, 989 (1992)) and kinase insert domain-containing receptor (referred to as "KDR" hereinafter) (WO 92/14748; *Proc. Natl. Acad. Sci., USA*, 88, 9026 (1991); *Biochem. Biophys. Res. Comm.*, 187, 1579 (1992); WO 94/11499), which belong to the receptor type tyrosine kinase family. Each of Flt-1 and KDR is a membrane protein of 180 to 200 kilodalton in molecular weight which has an extracellular domain consisting of 7 immunoglobulin-like regions and an intracellular domain consisting of a tyrosine kinase region. It has been reported that VEGF specifically binds to Flt-1 and KDR at KD values of 20 pM and 75 pM and that Flt-1 and KDR are expressed in vascular endothelial cells in a specific manner (*Proc. Natl. Acad. Sci., USA*, 90, 7533 (1993); *Proc. Natl. Acad. Sci., USA*, 90, 8915 (1993)). With regard to Flt-1 in various diseases, it has been reported that, in comparison with vascular endothelial cells in normal tissues, expression of Flt-1 mRNA increases in tumor vascular endothelial cells of human glioblastoma tissues (*Nature*, 359, 845 (1992)) and tumor vascular endothelial cells of human digestive organ cancer tissues (*Cancer Research*, 53, 4727 (1993)). Additionally, it has been reported that expression of Flt-1 mRNA is observed by *in situ* hybridization in vascular endothelial cells of joints of patients with rheumatoid arthritis (*J. Experimental Medicine*, 180, 341 (1994)). These results strongly suggest that a VEGF/VEGF receptor Flt-1 system plays an important role in tumor angiogenesis. Although it has been reported that VEGF binds to Flt-1 and the intracellular domain is auto-phosphorylated (*Science*, 255, 989 (1992)), the detailed function of the receptor mechanism is still unclear. However, it has been discovered that knock out mice in which the Flt-1 gene was destroyed die after a fetal age of 8.5 to 9.5 days due to abnormal blood vessel construction caused by abnormal morphology of vascular endothelial cells during blood island formation in the early stage of development and subsequent angiogenesis. This had led to an assumption that Flt-1 has a function essential for the tube formation of vascular endothelial cells in angiogenesis (*Nature*, 376, 66 (1995)).

In view of the above, it is expected that an antibody which can inhibit biological activities of VEGF through its binding to VEGF receptor Flt-1 will be useful for the diagnosis or treatment of diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity and psoriasis. However, an anti-VEGF receptor Flt-1 monoclonal antibody which can detect cells in which VEGF receptor Flt-1 is expressed and anti-VEGF receptor Flt-1 monoclonal antibody which can inhibit biological activities of VEGF has not been described in the art.

DISCLOSURE OF THE INVENTION

Concern has been directed toward the development of a method which is useful for the diagnosis or treatment of diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity and psoriasis. Although nothing has been reported on the anti-human VEGF receptor Flt-1 monoclonal antibody, it is considered that detection of the regions of angiogenesis and inhibition of angiogenesis by the use of an anti-human VEGF receptor Flt-1 monoclonal antibody will be useful for the diagnosis and treatment of these diseases. Consequently, development of anti-human VEGF receptor Flt-1 monoclonal antibody has been expected.

The present invention relates to a monoclonal antibody which specifically reacts with human VEGF receptor Flt-1. The present invention relates to a monoclonal antibody that recognizes an epitope which is present in a region of the 1st to 750th positions from the N-terminal amino acid (including a signal sequence) of human VEGF receptor Flt-1, particularly an epitope which is present in a region of the 1st to 338th positions from the N-terminal amino acid (including a signal sequence) of human VEGF receptor Flt-1. The present invention relates to a monoclonal antibody which specifically reacts with human VEGF receptor Flt-1 by immunocyte staining and to a monoclonal antibody which inhibits binding of human VEGF to human VEGF receptor Flt-1 and also inhibits biological activities of human VEGF. Examples of th monoclonal antibody which specifically reacts with human VEGF receptor Flt-1 by immunocyte staining include monoclonal antibody KM1730 belonging to the mouse IgG1 subclass, monoclonal antibody KM1731 belonging to the mouse IgG2a subclass, monoclonal antibody KM1732 belonging to the mouse IgG1 subclass, monoclonal antibody KM1748 belonging to the mouse IgG2b subclass and monoclonal antibody KM1750 belonging to the mouse IgG2b subclass. Examples of the monoclonal antibody which inhibits binding of human VEGF to human VEGF receptor Flt-1 and also inhibits biological activities of a human VEGF include monoclonal antibody KM1732 belonging to the mouse IgG1 subclass, monoclonal antibody KM1748 belonging to the mouse IgG2b subclass and monoclonal antibody KM1750 belonging to the mouse IgG2b subclass. Also, the present invention also relates to hybridoma KM1730 (FERM

BP-5697) which produces th monoclonal antibody KM1730, hybridoma KM1731 (FERM BP-5718) which produces th monoclonal antibody KM1731, hybridoma KM1732 (FERM BP-5698) which produces the monoclonal antibody KM1732, hybridoma KM1748 (FERM BP-5699) which produces the monoclonal antibody KM1748, and hybridoma KM1750 (FERM BP-5700) which produces the monoclonal antibody KM1750.

Furthermore, the present invention relates to, using the monoclonal antibody of the present invention, a method for immunologically detecting human VEGF receptor Flt-1, a method for immunologically detecting cells in which human VEGF receptor Flt-1 is expressed on the surface thereof, and a method for immunologically detecting and determining soluble human VEGF receptor Flt-1. Moreover, the present invention relates to, using the monoclonal antibody of the present invention, a method for inhibiting binding of human VEGF to human VEGF receptor Flt-1, and a method for inhibiting biological activities of human VEGF.

The present invention relates to a diagnostic agent of diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity, psoriasis, and the like.

The inventors of the present invention have found that anti-human VEGF receptor Flt-1 monoclonal antibody capable of recognizing an epitope present in a region of the 1st to 338th positions from the N-terminal amino acid of human VEGF receptor Flt-1 can specifically react with the human VEGF receptor Flt-1 by immunocyte staining, and that biological activities of human VEGF can be inhibited by the inhibition of binding of VEGF to anti-VEGF receptor Flt-1. Diagnosis and treatment of the above-described diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, prematurity retinopathy and psoriasis, can be carried out by using these monoclonal antibodies.

Consequently, the present invention provides antibodies which specifically react with human VEGF receptor Flt-1. With regard to the monoclonal antibody of the present invention, a monoclonal antibody is provided that recognizes an epitope which is present in a region of the 1st to 750th positions from the N-terminal amino acid (including a signal sequence) of human VEGF receptor Flt-1, particularly an epitope which is present in a region of the 1st to 338th positions from the N-terminal amino acid (including a signal sequence) of human VEGF receptor Flt-1, and also specifically reacts with human VEGF receptor Flt-1 by immunocyte staining. Also, the present invention provides a monoclonal antibody which inhibits binding of human VEGF to human VEGF receptor Flt-1 and also inhibits biological activities of the human VEGF. Examples of the monoclonal antibody which recognizes the epitope and also specifically reacts with human VEGF receptor Flt-1 by immunocyte staining include monoclonal antibody KM1730 produced by the hybridoma KM1730 (FERM BP-5697), monoclonal antibody KM1731 produced by the hybridoma KM1731 (FERM BP-5718), monoclonal antibody KM1732 produced by the hybridoma KM1732 (FERM BP-5698), monoclonal antibody KM1748 produced by the hybridoma KM1748 (FERM BP-5699), and monoclonal antibody KM1750 produced by the hybridoma KM1750 (FERM BP-5700). Examples of the monoclonal antibody which inhibits binding of human VEGF to human VEGF receptor Flt-1 and also inhibits biological activities of human VEGF include monoclonal antibody KM1732 produced by the hybridoma KM1732 (FERM BP-5698), monoclonal antibody KM1748 produced by the hybridoma KM1748 (FERM BP-5699), and monoclonal antibody KM1750 produced by the hybridoma KM1750 (FERM BP-5700).

The monoclonal antibody of the present invention may be any antibody, so long as it specifically reacts with human VEGF receptor Flt-1, but those which are established by the following production method can be cited as preferred examples. That is, anti-human VEGF receptor Flt-1 monoclonal antibody can be obtained by preparing human VEGF receptor Flt-1 protein as an antigen, immunizing an animal capable of providing a hybridoma with the antigen, such as mouse, rat, hamster, rabbit or the like, thereby inducing plasma cells having the antigen specificity, preparing a hybridoma capable of producing the monoclonal antibody through fusion of the cells with a myeloma cell line and subsequently culturing the hybridoma.

The production method of the anti-human VEGF receptor Flt-1 antibody of the present invention is described below.

1. Production method of anti-human VEGF receptor Flt-1 monoclonal antibody

(1) Preparation of antigen

Examples of the substance useful as the antigen for the preparation of the anti-human VEGF receptor Flt-1 monoclonal antibody include cells in which human VEGF receptor Flt-1 is expressed on the cell surface or a cell membrane fraction thereof, soluble human VEGF receptor Flt-1 protein having an extracellular region of different length and a fusion protein of the protein with Fc region of the antibody. As the cells capable of expressing human VEGF receptor Flt-1 on the cell surface, NIH3T3-Flt-1 cells (*Oncogene*, 10, 135 (1995)) can be exemplified. In a method for expressing the antigen as soluble human VEGF receptor Flt-1 protein having an extracellular region of different length or a fusion protein of the protein with Fc region of the antibody, the whole length or a partial fragment of cDNA which encodes human VEGF receptor Flt-1 (*Oncogene*, 5, 519 (1990); *Abstract of Papers, the 18th Annual meeting of Japan Molecu-*

lar Biology Society, 2P-227 (December, 1995)) is inserted into a downstream site of the promoter of an appropriate vector, the thus constructed recombinant vector is inserted into host cells and the thus obtained human VEGF receptor Flt-1 expression cells are cultured in an appropriate medium, thereby producing the whole length of a partial fragment of human VEGF receptor Flt-1 in the cells or culture supernatant as such or as a fusion protein.

As the host cells, any one of bacteria, yeast, animal cells, insect cells and the like can be used so long as they can express th gene of interest. Examples of th bacteria include the genus *Escherichia*, the genus *Bacillus* and the lik , such as *Escherichia coli*, *Bacillus subtilis* and the like. Examples of the yeast include *Saccharomyces cerevisiae*, *Schizosaccharomyces pompe* and the like. Examples of the animal cells include namalwa cells which are human cells, COS cells which are monkey cells and CHO cells which are Chinese hamster cells. Examples of the insect cells include Sf9 and Sf21 (produced by Pharmingen), High Five (produced by Invitrogen) and the like.

When a bacterium such as *Escherichia coli* is used as the host, the expression vector may be preferably constructed with a promoter, a ribosome binding sequence, the DNA of the present invention, a transcription termination sequence and, as occasion demands, a promoter controlling sequence. Examples include commercially available pGEX (produced by Pharmacia), pET System (produced by Novagen) and the like.

With regard to the method for introducing the recombinant vector into a bacterium, any one of the known methods for introducing DNA into bacteria, such as a method in which calcium ion is used (*Proc. Natl. Acad. Sci., USA*, 69, 2110-2114 (1972)), a protoplast method (Japanese Published Unexamined Patent Application No. 2483942/91), and the like can be used.

When yeast is used as the host, YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), or the like is used as the expression vector.

With regard to the method for introducing the recombinant vector into yeast, any one of the known methods for introducing DNA into yeast, such as an electroporation method (*Methods. Enzymol.*, 194, 182-187 (1990)), a spheroplast method (*Proc. Natl. Acad. Sci., USA*, 84, 1929-1933 (1978)), a lithium acetate method (*J. Bacteriol.*, 153, 163-168 (1983)), and the like can be used.

When animal cells are used as the host, pAGE107 (Japanese Published Unexamined Patent Application No. 22979/88; *Cytotechnology*, 3, 133 (1990)), pAGE103 (*J. Biochem.*, 101, 1307 (1987)), and the like can be exemplified as the useful expression vector.

Any promoter capable of effecting the expression in animal cells can be used. Examples include the promoter of IE (immediate early) gene of cytomegalovirus (CMV), the SV40 promoter, the metallothionein promoter and the like. Furthermore, the enhancer of the IE gene of human CMV may be used together with the promoter.

With regard to the method for the introduction of the recombinant vector into animal cells, any one of the known methods for introducing DNA into animal cells, such as an electroporation method (*Cytotechnology*, 3, 133 (1990)), a calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), a lipofection method (*Proc. Natl. Acad. Sci., USA*, 84, 7413 (1987)) and the like can be used.

When insect cells are used as the host, the protein can be expressed by the known method described in, for example, *Current Protocols in Molecular Biology*, Supplement 1-34 and *Baculovirus expression vectors*, A laboratory manual. That is, the recombinant gene introducing vector and baculovirus described in the following are simultaneously introduced into insect cells to obtain a recombinant virus in the insect cell culture supernatant and then the insect cells are infected with the thus obtained recombinant virus to obtain protein-expressing insect cells.

Examples of the gene introducing vector include pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen), and the like.

Examples of the baculovirus include Autograph californica nuclear polyhedrosis virus with which insects of the family *Barathra* are infected.

With regard to the method for the simultaneous introduction of the above-described recombinant gene introducing vector and the above-described baculovirus into insect cells for the preparation of the recombinant virus, calcium phosphate method (Japanese Published Unexamined Patent Application No. 227075/90), lipofection method (*Proc. Natl. Acad. Sci., USA*, 84, 7413 (1987)) and the like can be exemplified.

Alternatively, the protein of interest can be produced by preparing a recombinant baculovirus making use, for example, of BaculoGold Starter Kit manufactured by Pharmigen and then infecting the above-described insect cells, such as Sf9, Sf21, High Five, or the like, with the recombinant virus (*Bio/Technology*, 6, 47 (1988)).

With regard to the gene expression method, techniques, such as secretion production, fusion protein expression and the like have been developed, and each of these every methods can be used. For example, gene expression can be produced in accordance with the method described in *Molecular Cloning* 2nd edition, Cold Spring Harbor Lab. Press, New York (1989), or by direct expression.

The whole length or a partial fragment of a human VEGF receptor Flt-1 can be produced as such or as a fusion protein thereof by culturing a transformant obtained in the above-described manner in a culture medium, thereby effecting formation and accumulation of the protein of the present invention in the resulting culture mixture, and then collecting the protein from the culture mixture.

Culturing of the transformant of the present invention in a culture medium is carried out in accordance with a usual method which is used in the culturing of respective host cells.

With regard to the medium for use in the culturing of the transformant obtained using a microorganism, such as *Escherichia coli*, yeast, or the like, as the host, either a natural medium or a synthetic medium may be used, so long as it contains materials which can be assimilated by th microorganism, such as carbon sources, nitrogen sources, inorganic salts, and the like, and can perform culturing of the transformant efficiently (*Molecular Cloning 2nd edition*, Cold Spring Harbor Lab. Press, New York (1989)). The culturing is carried out generally under aerobic conditions, such as a shaking culture, submerged agitation aeration culture, or the like, at 15 to 40°C for 16 to 96 hours. During the culturing, the pH is controlled to 3.0 to 9.0. Adjustment of the pH is carried out using an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia, and the like. As occasion demands, antibiotics, such as ampicillin, tetracycline, and th like may be added to the medium during the culturing.

With regard to the medium for use in the culturing of a transformant obtained using animal cells as the host, RPMI 1640 medium, Eagle's MEM medium or any one of these media further supplemented with fetal calf serum may be used. The culturing is carried out generally at 35 to 37°C for 3 to 7 days in the presence of 5% CO_2. As occasion demands, antibiotics, such as kanamycin, penicillin, and the like may be added to the medium during the culturing.

With regard to the medium for use in the culturing of a transformant obtained using insect cells as the host, TNM-FH medium (produced by Pharmingen), Sf900IISFM (produced by Life Technologies), ExCell400 or ExCell405 (both produced by JRH Biosciences), or the like may be used. The culturing is carried out generally at 25 to 30°C for 1 to 4 days, and gentamicin and the like antibiotics may be added to the medium during the culturing as occasion demands.

Although media for the culturing of animal cells and insect cells contain serum, it is desirable to use a serum-free medium in order to efficiently purify the whole length or a partial fragment of human VEGF receptor Flt-1 as such or as a fusion protein.

When the whole length or a partial fragment of human VEGF receptor Flt-1 is accumulated inside the host cells as such or as a fusion protein, the cells after completion of the culturing are collected by centrifugation, suspended in an aqueous buffer and then disrupted using ultrasonic oscillator, French press, or the like, and subsequently collecting the protein from a supernatant fluid prepared by centrifuging the thus disrupted cells.

Also, when an insoluble body is formed inside the cells, the insoluble body is solubilized using a protein denaturing agent and then higher-order structure of the protein is formed by diluting or dialyzing the thus solubilized protein in or against a solution which does not contain the protein denaturing agent or contains the agent but in such a low concentration that the protein is not denatured.

When the whole length or a partial fragment of human VEGF receptor Flt-1 is secreted outside the cells as such or as a fusion protein, the expressed protein can be collected from the culture supernatant. The isolation and purification can be carried out by employing separation means, such as solvent extraction, fractional precipitation with organic solvents, salting out, dialysis, centrifugation, ultracentrifugation, ion exchange chromatography, gel filtration chromatography, hydrophobic chromatography, affinity chromatography, reverse phase chromatography, crystallization, electrophoresis, and the like, alone or in combination.

(2) Immunization of animals and preparation of antibody producing cells

Although any one of animals, such as mice, rats, hamsters, rabbits, and the like, can be used in the immunization, so long as a hybridoma can be prepared, an example in which mice and rats are used is described in this invention. A mouse or rat of 3 to 20 weeks of age is immunized with the protein obtained in the above step 1-(1) as the antigen, and antibody producing cells are collected from the spleen, lymph node or peripheral blood of the animal. The immunization is carried out by administering the antigen several times through subcutaneous, intravenous or intraperitoneal injection together with an appropriate adjuvant. As the adjuvant, a complete Freund's adjuvant or a combination of aluminum hydroxide gel with pertussis vaccine can be exemplified. A blood sample is collected from the fundus of the eye or caudal vein of the animal 3 to 7 days after each administration, the sample is tested, for example, by enzyme immunoassay ((Enzyme-linked Immunosorbent Assay (ELISA), published by Igaku Shoin, (1976)) as to whether it is reactive with the antigen used, namely soluble human VEGF receptor Flt-1 or NIH3T3 cells in which human VEGF receptor Flt-1 is expressed on the cell surface, and then a mouse or rat showing sufficient antibody titer in their sera is submitted for use as the supply source of antibody producing cells. On the 3rd to 7th day after final administration of the antigen, the spleen is excised from the immunized mouse to carry out fusion of the spleen cells with myeloma cells in accordance with the known method (*Antibodies -A Laboratory Manual*, Cold Spring Harbor Laboratory, (1988); referred to as "*Antibodies - A Laboratory Manual*" hereinafter).

(3) Preparation of myeloma cells

As the myeloma cells, any myeloma cells capable of growing *in vitro* may be used, which include established cells

obtained from mouse, such as 8-azaguanine-resistant mouse (BALB/c) myeloma cell line P3-X63Ag8-U1 (P3-U1) (G. Kohler *et al.*, *Europ. J. Immunol*, 6, 511 (1976)), SP2/O-Ag14 (SP-2) (M. Shulman *et al.*, *Nature*, 276, 269 (1978)), P3-X63-Ag8653 (653) (J.F. Kearney *et al.*, *J. Immunol.*, 123, 1548 (1979)), P3-X63-Ag8 (X63) (G. Kohler *et al.*, *Nature*, 256, 495 (1975)), and the like. These cell lines are cultured and subcultured in accordance with the known method (*Antibodies - A Laboratory Manual*) and 2×10^7 or more of the cells are secured until cell fusion.

(4) Cell fusion

The antibody producing cells obtained in the above step (2) and the myeloma cells obtained in the above step (3) are washed, mixed with cell aggregating medium, polyethylene glycol-1000 (PEG-1000) or the like, to effect cell fusion and then suspended in a culture medium. For the washing of the cells, MEM medium or PBS (1.83 g of disodium hydrogen phosphate, 0.21 g of potassium dihydrogen phosphate, 7.65 g of sodium chloride, 1 liter of distilled water, pH 7.2) is used. In order to obtain the fused cells of interest selectively, HAT medium {normal medium (a medium prepared by adding glutamine (1.5 mM), 2-mercaptoethanol (5×10^{-5} M), gentamicin (10 µg/ml) and fetal calf serum (FCS) (10%, produced by CSL) to RPMI-1640 medium) further supplemented with hypoxanthine (10^{-4} M), thymidine (1.5×10^{-5} M) and aminopterin (4×10^{-7} M)} is used as the medium for suspending the fused cells.

After the culturing, a portion of the culture supernatant is sampled and tested, for example, by an enzyme immunoassay method which will be described in the following step (5) to select wells which can specifically react with human VEGF receptor Flt-1 or a recombinant protein such as a fusion protein with human VEGF receptor Flt-1 described in the above step (1). Thereafter, cloning is carried out twice by limiting dilution analysis (using HT medium (a medium prepared by eliminating aminopterin from the HAT medium) for the first analysis and the normal medium for the second analysis), and a hybridoma which shows stable and high antibody titer is selected as the hybridoma capable of producing the anti-human VEGF receptor Flt-1 monoclonal antibody.

(5) Selection of anti-human VEGF receptor Flt-1 monoclonal antibody

Selection of a hybridoma capable of producing the anti-human VEGF receptor Flt-1 monoclonal antibody is carried out by the enzyme immunoassay method described below.

Enzyme immunoassay

Human VEGF receptor Flt-1 or a recombinant protein such as a fusion protein with the human VEGF receptor Flt-1 described in the above step 1-(1) is coated on an appropriate plate and allowed to react with a first antibody, namely a hybridoma culture supernatant or a purified antibody obtained in the following step 1-(6), and then with a second antibody, namely an anti-mouse immunoglobulin antibody or anti-rat immunoglobulin antibody labeled with biotin, an enzyme, a chemiluminescent substance, a radioactive compound or the like, and then a reaction suitable for the label used is carried out in order to select a sample which specifically reacts with human VEGF receptor Flt-1 as a hybridoma capable of producing anti-human VEGF receptor Flt-1 monoclonal antibody. Examples of the hybridoma include hybridomas KM1730, KM1731, KM1732, KM1748 and KM1750. The hybridomas KM1730, KM1732, KM1748 and KM1750, on October 8, 1996, and the hybridoma KM1731, on October 22, 1996, were deposited with National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology (Higashi 1-1-3, Tsukuba-shi, Ibaraki, Japan), and were assigned the designations as FERM BP-5697, FERM BP-5698, FERM BP-5699, FERM BP-5700 and FERM BP-5718, respectively.

(6) Preparation of monoclonal antibody

The anti-human VEGF receptor Flt-1 monoclonal antibody-producing hybridoma cells obtained in the above-described step 1-(3) are administered by intraperitoneal injection into 8- to 10-week-old mice or nude mice treated with pristane (by intraperitoneal administration of 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane) followed by 2 weeks of feeding) at a dose of 2×10^7 to 5×10^6 cells/animal. The hybridoma causes ascites tumor in 10 to 21 days. The ascitic fluid is collected from the mice or nude mice, centrifuged, subjected to salting out with 40 to 50% saturated ammonium sulfate or to caprylic acid precipitation and then passed through a DEAE-Sepharose column, protein A column or Cellulofine GSL 2000 (produced by Seikagaku Kogyo) to collect an IgG or IgM fraction to give a purified monoclonal antibody.

The subclass of the purified monoclonal antibody can be determined using a mouse monoclonal antibody typing kit or a rat monoclonal antibody typing kit. The amount of protein can be determined by the Lowry method or by calculation based on the optical density at 280 nm.

Furthermore, the present invention relates to, using the monoclonal antibody of the present invention, a method for

immunologically detecting human VEGF receptor Flt-1 or cells in which human VEGF receptor Flt-1 is expressed on the surface thereof, a method for immunologically detecting and determining soluble human VEGF receptor Flt-1, and a method for inhibiting binding of a human VEGF to human VEGF receptor Flt-1 or biological activities of human VEGF.

Moreover, the present invention relates to a diagnostic agent for diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity, psoriasis, and the like.

The methods for detecting and determining human VEGF receptor Flt-1 are described below.

2. Detection and determination of human VEGF receptor Flt-1 using anti-human VEGF receptor Flt-1 monoclonal antibody

Examples of the methods, using the monoclonal antibody of the present invention, for immunologically detecting human VEGF receptor Flt-1 or a cell in which human VEGF receptor Flt-1 is expressed on the surface thereof and for immunologically detecting and determining soluble human VEGF receptor Flt-1 include immunocyte staining, Western blotting, sandwich ELISA, and the like. These methods are described below.

(1) Immunocyte staining using monoclonal antibody

Firstly, the cells in which human VEGF receptor Flt-1 is expressed on the cell surface are prepared. Suspending cells as such or adherent cells after detachment of the cells using trypsin-EDTA are suspended, for example, in a buffer solution for immunocyte staining use (PBS containing 1% BSA, 0.02% EDTA and 0.05% sodium azide) and dispensed in 1×10^5 to 2×10^6 portions. A culture supernatant of the anti-human VEGF receptor Flt-1 monoclonal antibody-producing hybridoma obtained in the above-described step 1-(4), the purified monoclonal antibody obtained in the above-described step 1-(6) or the monoclonal antibody labeled with biotin by a known method (*Enzyme Antibody Method*: published by Gakusai Kikaku 1985) is diluted with the buffer solution for immunocyte staining use or the buffer solution for immunocyte staining use further supplemented with 10% animal serum to a concentration of 0.1 to 50 µg/ml and dispensed in 20 to 500 µl portions to carry out the reaction under cooling for 30 minutes. When the culture supernatant of the anti-human VEGF receptor Flt-1 monoclonal antibody-producing hybridoma obtained in the above-described step 1-(4) or the purified monoclonal antibody obtained in the above-described step 1-(6) is used in the reaction, the cells are washed with the buffer solution for immunocyte staining use and then an anti-mouse immunoglobulin antibody or anti-rat immunoglobulin labeled with fluorescence dye, such as FITC, phycoerythrin, or the like, which is dissolved in the buffer solution for immunocyte staining use to a concentration of about 0.1 to 50 µg/ml, is dispensed in 50 to 500 µl portions to carry out the reaction under cooling for 30 minutes. When the monoclonal antibody labeled with biotin is used in the reaction, streptoavidin is dispensed in 50 to 500 µl portions and then the reaction is carried out under cooling in the dark for 30 minutes. After completion of the reaction, the cells are thoroughly washed with the buffer solution for immunocyte staining use and analyzed by a cell sorter.

(2) Detection of human VEGF receptor Flt-1 by Western blotting

Cell membrane components are prepared from cells in which human VEGF receptor Flt-1 is expressed, such as human VEGF receptor Flt-1-expressing NIH3T3 cells (referred to as "NIH3T3-Flt-1" hereinafter), and from control cells such as NIH3T3 cells (referred to as "NIH3T3-Neo" hereinafter) (*Oncogene*, 10, 135 (1995)), and the membrane compon nts are subjected to electrophoresis by the SDS-PAGE method under reducing conditions in an amount of 0.1 to 30 µl as protein per lane. The thus treated proteins are transferred on a PVDF membrane and allowed to react with PBS containing 1% BSA at room temperature for 30 minutes to effect blocking. They are allowed to react with the culture supernatant of the anti-human VEGF receptor Flt-1 monoclonal antibody-producing hybridoma obtained in the above-described step 1-(4) or the purified monoclonal antibody obtained in the above-described step 1-(6), washed with PBS containing 0.05% Tween and then allowed to react with peroxidase-labeled goat anti-mouse IgG at room temperature for 2 hours. After washing with PBS containing 0.05% Tween, bands to which the anti-human VEGF receptor Flt-1 monoclonal antibody is linked are detected using ECL™ Western blotting detection reagents (produced by Amersham) or the like.

(3) Determination of soluble VEGF receptor Flt-1 using monoclonal antibody

As a first antibody, the purified monoclonal antibody obtained in the above-described step 1-(6) is coated on an appropriate plate and allowed to react with 0.056 to 10,000 ng/ml of the purified soluble anti-human VEGF receptor Flt-1 monoclonal antibody-producing hybridoma obtained in the above-described step 1-(1), or with a sample, such as human serum or the like. After through washing of the plate, this is allowed to react with a second antibody, namely a

monoclonal antibody labeled with biotin, an enzym , a chemiluminescence substance, a radioactive compound or the like, which is one of the purified monoclonal antibodies obtained in the above-described step 1-(6) but recognizes different epitope from that of the monoclonal antibody used as the first antibody, and then the reaction suitable for th label used is carried out. A calibration curve is prepared based on the reactivity for the purified soluble VEGF receptor Flt-1, and the concentration of the soluble VEGF receptor Flt-1 in the samples is calculated.

The inhibition methods of the bonding of human VEGF to human VEGF receptor Flt-1 and of the biological activities of human VEGF are exemplified.

(4) Test on the inhibition of the binding of VEGF to VEGF receptor Flt-1 using monoclonal antibody

Methanol is dispensed in 100 µl portions into wells of a 96 well MultiScreen-IP Plate (produced by Millipore) to give hydrophilic nature to the PVDF membrane on the bottom of the plate. After washing with water, human VEGF receptor Flt-1 or a recombinant protein such as a fusion protein with human VEGF receptor Flt-1 is diluted to a concentration of 0.1 to 10 µg/ml, dispensed in 50 µl/well portions and then allowed to stand overnight at 4°C to effect its adsorption. After washing, PBS containing 1% bovine serum albumin (BSA) is dispensed in 100 µl/well portions and the reaction is carried out at room temperature for 1 hour to effect blocking of any remaining active groups. After washing with PBS, the culture supernatant of the anti-human VEGF receptor Flt-1 monoclonal antibody-producing hybridoma obtained in the above-described step 1-(4) or the purified monoclonal antibody obtained in the above-described step 1-(6) is dispensed in 50 µl/well portions and then 0.1 to 10 ng/ml of ^{125}I-labeled VEGF (produced by Amersham) is dispensed in 50 µl/well portions, subsequently carrying out the reaction at room temperature for 1.5 hours. After washing with 0.05% Tween-PBS, the wells are dried at 50°C, and a scintillator is dispensed in 20 to 100 µl/well portions to measure the radioactivity of the ^{125}I-labeled VEGF linked to each well using Top Count (produced by Packard) or the like.

(5) Test on the inhibition of the binding of VEGF to VEGF receptor Flt-1-expressing cells using monoclonal antibody

PBS containing 1% bovine serum albumin (BSA) is dispensed in 100 µl portions into wells of a 96 well MultiScreen-HV Plate (produced by Millipore), the reaction is carried out at room temperature for 1 hour to effect blocking of the active groups in wells and then NIH3T3-Flt-1 cells suspended in 1% BSA-PBS containing 0.05% NaN_3 is dispensed in 1×10^4 to 1×10^5/well portions. After washing with 1% BSA-PBS, the culture supernatant of the anti-human VEGF receptor Flt-1 monoclonal antibody-producing hybridoma obtained in the above-described step 1-(4) or the purified monoclonal antibody obtained in the above-described step 1-(6) is dispensed in 50 µl/well portions and then 0.1 to 10 ng/ml of ^{125}I-labeled VEGF (produced by Amersham) is dispensed in 50 µl/well portions, subsequently carrying out the reaction at room temperature for 1.5 hours. After washing with PBS, the wells are dried at 50°C, and a scintillator is dispensed in 20 to 100 µl/well portions to measure the radioactivity of the ^{125}I-labeled VEGF linked to each well using Top Count (produced by Packard) or the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing construction steps of plasmid pVL1393/Flt 3N.

Fig. 2 is a graph showing construction steps of plasmid pVL1393/Flt 7N.

Fig. 3 is a graph showing patterns of SDS polyacrylamide electrophoresis (a 5 to 20% gradient gel was used) of purified Flt-1 7N and Flt-1 3N. Starting from the left side, electrophoresis patterns of molecular weight markers, Flt-1 3N and Flt-1 7N are shown respectively. The electrophoresis was carried out under reducing conditions.

Fig. 4 is a graph showing results of the analysis of the effect of soluble human VEGF receptors Flt-1 7N and Flt-1 3N to inhibit binding of a ^{125}I-human VEGF to a plate-coated soluble human VEGF receptor Flt-1 7N.

Fig. 5 is a graph showing results of the examination of the binding reactivity of anti-human VEGF receptor Flt-1 monoclonal antibody by enzyme immunoassay.

Fig. 6 is a graph showing results of the examination of the activity of anti-human VEGF receptor Flt-1 monoclonal antibody to inhibit binding of VEGF to human VEGF receptor Flt-1.

Fig. 7 is a graph showing results of the examination of the activity of anti-human VEGF receptor Flt-1 monoclonal antibodies KM1732, KM1748 and KM1750 to inhibit binding of human VEGF to human VEGF receptor Flt-1.

Fig. 8 is a graph showing results of the examination of the activity of anti-human VEGF receptor Flt-1 monoclonal antibodies KM1732, KM1748 and KM1750 to inhibit binding of human VEGF to human VEGF receptor Flt-1-expressing cells.

Fig. 9 is a graph showing results of the flow cytometry analysis of the reactivity of anti-human VEGF receptor Flt-1 monoclonal antibodies KM1730, KM1731, KM1732, KM1748 and KM1750 with human VEGF receptor Flt-1-expressing cells NIH3T3-Flt-1 and control cells NIH3T3-Neo cells.

Fig. 10 is a graph showing results of the examination of the reactivity of anti-human VEGF receptor Flt-1 mono-

clonal antibody KM1737 with human VEGF receptor Flt-1 by Western blotting. Lane 1 shows Western blotting pattern of NIH3T3-Flt-1 cells and lan 2 shows the pattern of NIH3T3-Neo cells.

Fig. 11 is a graph showing results of the examination of the determination system of soluble human VEGF receptors Flt-1 3N and Flt-1 7N, carried out using anti-human VEGF receptor Flt-1 monoclonal antibodies KM1732 and biotinated KM1730.

Fig. 12 is a graph showing results of the flow cytometry analysis of the reactivity of anti-human VEGF receptor Flt-1 monoclonal antibody with human vascular endothelial cells HUVEC.

Fig. 13 is a graph showing results of the flow cytometry analysis of the reactivity of anti-human VEGF receptor Flt-1 monoclonal antibody with human vascular endothelial cells HUVEC under a VEGF non-stimulation or stimulation condition.

Fig. 14 is a graph showing results of the analysis on the changes in the expression quantity of human VEGF receptor Flt-1 in human vascular endothelial cells HUVEC under a VEGF non-stimulation or stimulation condition. The expression quantity of Flt-1 is shown as a relative reaction value of anti-human VEGF receptor Flt-1 monoclonal antibody KM1730 when the reactivity of a control antibody is defined as 1.

BEST MODE FOR CARRYING OUT THE INVENTION

Example 1

1. Preparation of antigen

(1) Construction of soluble human VEGF receptor Flt-1 3N expression vector

A vector was prepared in the following manner, for use in the expression of a soluble human VEGF receptor Flt-1 fragment (referred to as "soluble human VEGF receptor Flt-1 3N" hereinafter) which corresponds to a region of the 1st to 338th positions (including a signal sequence) from the N-terminal amino acid of human VEGF receptor Flt-1. The soluble human VEGF receptor Flt-1 3N corresponds to the N-terminal side three immunoglobulin-like regions of the extracellular domain of the soluble human VEGF receptor Flt-1.

A cDNA clone flt#3-7 (M. Shibuya *et al.*, *Oncogene*, 5, 519, 1990) which contains whole length cDNA coding for the human VEGF receptor Flt-1 was partially digested with restriction enzymes *Eco*RI and *Taq*I to collect a 1,263 bp *Eco*RI-*Taq*I DNA fragment from the 5'-end, and the thus collected fragment was inserted into the 5' side *Eco*RI site and 3' side *Not*I site downstream of the transcription initiation point of the polyhedrin gene of a baculovirus gene recombinant vector pVL1393 plasmid (produced by Invitrogen) using a *Taq*I-*Not*I adapter into which a termination codon had been artificially introduced (a synthetic DNA fragment having the nucleotide sequences shown in the SEQ ID NO: 1 and NO: 2), thereby obtaining soluble human VEGF receptor Flt-1 3N expression vector pVL1393/Flt 3N (Fig. 1).

(2) Construction of soluble human VEGF receptor Flt-1 7N expression vector

A vector was prepared in the following manner, for use in the expression of a soluble human VEGF receptor Flt-1 fragment (referred to as "soluble human VEGF receptor Flt-1 7N" hereinafter) which corresponds to a region of the 1st to 750th positions (including a signal sequence) from the N-terminal amino acid of human VEGF receptor Flt-1. The soluble human VEGF receptor Flt-1 7N corresponds to the seven immunoglobulin-like regions of the extracellular domain of the soluble human VEGF receptor Flt-1.

A 2.5 unit portion of Taq polymerase was added to 100 μl of 0.001% (w/v) gelatin solution of 10 mM $MgCl_2$ containing 10 pmol of primers having the nucleotide sequences shown in SEQ ID NO:3 and NO: 4, 10 ng of flt#3-7 clone (*Oncogene*, 5, 519, (1990)) DNA and 10 mM deoxynucleotide triphosphates. The polymerase chain reaction (PCR) was repeated 30 times in which one reaction consisted, after pretreatment at 95°C for 5 minutes, of treatments at 95°C for 90 seconds, at 50°C for 90 seconds and finally at 72°C for 90 seconds, subsequently collecting a DNA fragment. The DNA fragment was digested with *Hind*III (the 1893 bp position in the flt#3-7 clone) and *Not*I to obtain a 610 bp *Hind*III-*Not*I DNA fragment, namely a DNA fragment containing a 1894-2499 bp fragment of the flt#3-7 clone, termination codon and *Not*I recognition sequence. Next, the flt#3-7 clone was digested with restriction enzymes *Eco*RI and *Hind*III to collect an *Eco*RI-*Hind*III fragment of 1893 bp from the 5'-end. The 610 bp *Hind*III-*Not*I DNA fragment and the 1893 bp *Eco*RI-*Hind*III fragments were then inserted into the 5' side *Eco*RI site and 3' side *Not*I site downstream of the transcription initiation point of the polyhedrin gene of a baculovirus gene recombinant vector pVL1393 plasmid, thereby preparing soluble human VEGF receptor Flt-1 7N expression vector pVL1393/Flt 7N (Fig. 2).

(3) Preparation of recombinant virus for use in the expression of soluble human VEGF receptor Flt-1 in insect cells

For the production of protein by insect cells, it is necessary to prepare a recombinant virus into which a gene of interest is integrated, and the preparation process consists of a step in which a cDNA molecule coding for a protein of interest is inserted into a special plasmid, which is called a transfer vector, and a subsequent step in which a wild type virus and the transfer vector are co-transfected into insect cells to obtain a recombinant virus by homologous recombination. These steps were carried out in the following manner using BaculoGold Starter Kit manufactured by Pharmigen (product no. PM-21001K) in accordance with the manual.

A recombinant baculovirus was prepared in the following manner by introducing a filamentous baculovirus DNA (BaculoGold baculovirus DNA, produced by Pharmigen) and the thus prepared transfer vector DNA into insect cells Sf9 (produced by Pharmigen) which had been cultured using TMN-FH insect medium (produced by Pharmigen), using a lipofectin method (*Protein, Nucleic Acid, Enzyme*, 37, 2701 (1992)).

A 1 µg portion of pVL1393/Flt7N prepared in the above step (2) or pVL1393/Flt3N prepared in the above step (1) and 20 ng of filamentous baculovirus DNA were dissolved in 12 µl of distilled water, the solution was mixed with a mixture of 6 µl lipofectin and 6 µl distilled water and then the resulting mixture was allowed to stand at room temperature for 15 minutes. Separately from this, 1×10^6 of Sf9 cells were suspended in 2 ml of Sf900-II medium (produced by Gibco) and put into a cell culture plastic Petri dish of 35 mm in diameter. To this was added whole volume of the just described solution of plasmid DNA, filamentous baculovirus DNA and lipofectin mixture, followed by 3 days of culturing at 27°C to collect 1 ml of the culture supernatant containing the recombinant virus. A 1 ml portion of Sf900-II medium was added to the resulting Petri dish and 3 days of culturing was carried out at 27°C to obtain an additional 1.5 ml of the recombinant virus containing culture supernatant.

Next, the thus obtained recombinant virus for use in the protein expression was grown in the following manner.

A 2×10^7 portion of Sf9 cells were suspended in 10 ml of Sf900-II medium, put into a 175 cm^2 flask (produced by Greiner) and allowed to stand at room temperature for 1 hour to effect adhesion of the cells to the flask. The supernatant fluid was subsequently discarded and 15 ml of fresh TMN-FH insect medium and a 1 ml portion of the recombinant virus containing culture supernatant described above were added and cultured for 3 days at 27°C. After the culturing, the supernatant fluid was centrifuged at 1,500 × g for 10 minutes to remove the cells, thereby obtaining a recombinant virus solution for use in the protein expression.

The titer of virus in the thus obtained recombinant virus solution was calculated by the method described in BaculoGold Starter Kit Manual (Pharmigen).

A 6×10^6 portion of Sf9 cells were suspended in 4 ml of Sf900-II medium, put into a cell culture plastic Petri dishes of 60 mm in diameter and allowed to stand at room temperature for 1 hour to effect adhesion of the cells to the dish. Next, the supernatant fluid was discarded, 400 µl of fresh Sf900-II medium and the above-described recombinant virus solution diluted 10,000 times with Sf900-II medium were added to the dish and allowed to stand at room temperature for 1 hour, the medium was removed and then 5 ml of a medium containing 1% low melting point agarose (Agarplaque Agarose, produced by Pharmigen) (prepared by mixing 1 ml of sterilized 5% Agarplaque plus agarose aqueous solution with 4 ml of TMN-FH insect medium and stored at 42°C) was poured into the dish. After standing at room temperature for 15 minutes, the dish was tied with a vinyl tape to prevent drying, put into a sealable plastic container and then subjected to 6 days of culturing at 27°C. A 1 ml portion of PBS containing 0.01% of Neutral Red was added to the dish to carry out the additional culturing for 1 day and then the number of the thus formed plaques was counted. By the above procedure, it was found that each of the recombinant virus solutions contained virus particles of about 1×10^7 plaque forming units (referred to as "PFU" hereinafter) per ml.

(4) Expression of soluble human VEGF receptors Flt-1 7N and Flt-1 3N in insect cells and purification thereof

Soluble human VEGF receptors Flt-1 7N and Flt-1 3N were obtained in the following manner. A 4×10^7 portion of High Five cells were suspended in 30 ml of EX-CELL™ 400 medium (produced by JRH Biosciences) contained in a 175 cm^2 flask (produced by Greiner) and allowed to stand at room temperature for 1 hour to effect adhesion of the cells to the flask. A 1 ml portion of a solution containing about 1 to 3×10^8 PFU/ml of recombinant virus particles obtained in the above step (3) from the transfer vectors pVL1393/Flt 7N and pVL1393/Flt 3N was added to the flask to carry out infection at room temperature for 2 hours. The culture supernatant was removed and 30 ml of fresh EX-CELL™ 400 medium was added to carry out 3 to 4 days of culturing at 27°C. After completion of the culturing, the culture supernatant was collected and centrifuged at 1,500 × g for 10 minutes to obtain a supernatant fluid.

A column was packed with about 60 ml of heparin-Sepharose CL-6B gel (produced by Pharmacia Biotech AB) and washed with 600 ml of 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 ml/minute. After the washing, 1,000 ml of the culture medium containing soluble human VEGF receptors Flt-1 7N and Flt-1 3N, which had been prepared in the above-described manner, was passed through the heparin-Sepharose CL-6B column at a flow rate of 0.5 ml/minute. After washing with 600 ml of 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 ml/minute, 600 ml of 20 mM Tris-HCl

(pH 7.5) buffer having a density gradient of 0 M to 1.1 M NaCl was passed through the column to carry out elution of the proteins adsorbed to the heparin-Sepharose, and the eluate was fractionated in 8 ml portions. Proteins contained in each fraction wer analyzed by SDS polyacrylamid gel electrophoresis (SDS-PAGE), and 60 to 80 ml of fractions containing soluble human VEGF receptors Flt-1 7N and Flt-1 3N were collected and concentrated using CentriPrep 10 (produced by Amicon). After the concentration, soluble human Flt-1 7N and Flt-1 3N were obtained as solutions of 5 ml and 13 ml, respectively (protein concentrations were 331 µg/ml and 204 µg/ml).

(5) Confirmation of the purity of soluble human VEGF receptors Flt-1 7N and Flt-1 3N

Purity of the thus purified soluble human VEGF receptors Flt-1 7N and Flt-1 3N was confirmed by SDS-PAGE. The SDS-PAGE was carried out in accordance with a known method (*Anticancer Research*, 12, 1121, 1992). Using a 5 to 20% gradient gel (produced by Atto) as the gel, electrophoresis of Flt-1 7N and Flt-1 3N, each 2 µg as protein per lane, was carried out under reducing conditions, and the resulting gel was stained with Coomassie Brilliant Blue. The results are shown in Fig. 3. Purity of Flt-1 7N and Flt-1 3N was found to be 95% or more.

(6) Purification of control antigen protein of soluble human VEGF receptors Flt-1 7N and Flt-1 3N

The control antigen protein (negative control protein) of soluble human VEGF receptors Flt-1 7N and Flt-1 3N was obtained in the following manner. A 4×10^7 portion of High Five cells were suspended in 30 ml of EX-CELL™ 400 medium (produced by JRH Biosciences) contained in a 175 cm^2 flask (produced by Greiner), allowed to stand at room temperature for 1 hour to effect adhesion of the cells to the flask and then cultured at 27°C for 3 to 4 days. After completion of the culturing, the culture supernatant was collected and centrifuged at 1,500 × g for 10 minutes to obtain a supernatant fluid.

A column was packed with about 20 ml of heparin-Sepharose CL-6B gel (produced by Pharmacia Biotech AB) and washed with 200 ml of 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 ml/minute. After the washing, 500 ml of the culture medium of High Five cells was passed through the heparin-Sepharose CL-6B column at a flow rate of 0.5 ml/minute. After washing with 200 ml of 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 ml/minute, 200 ml of 20 mM Tris-HCl (pH 7.5) buffer containing 1 M NaCl was passed through the column to carry out elution of the protein adsorbed to the heparin-Sepharose. The 1 M NaCl elution fraction was concentrated using CentriPrep 10 (produced by Amicon) to obtain 7 ml of the control antigen protein (867 µg/ml as protein concentration).

(7) Confirmation of human VEGF binding activity of soluble human VEGF receptors Flt-1 7N and Flt-1 3N

The human VEGF binding activity of soluble human VEGF receptors Flt-1 7N and Flt-1 3N was confirmed in the following manner.

Methanol was dispensed in 100 µl portions into wells of a 96 well Immobilon™-P Filtration Plate (produced by Millipore) to give a hydrophilic nature to the PVDF membrane on the bottom of the plate. After washing with water, the soluble human Flt-1 7N diluted to a concentration of 2 µg/ml was dispensed in 50 µl/well portions and allowed to stand overnight at 4°C to effect its adsorption. After washing, PBS containing 1% bovine serum albumin (BSA) was dispensed in 100 µl/well portions and 1 hour of the reaction was carried out at room temperature to effect blocking of the remained active groups. After washing with PBS, each of the purified soluble human VEGF receptors Flt-1 7N and Flt-1 3N obtained in the above-described step (4) was dispensed in 50 µl/well portions (final concentration, 1 to 1,000 ng/ml) and then ^{125}I-labeled human VEGF (final concentration, 3 ng/ml: produced by Amersham) was dispensed in 50 µl/well portions, subsequently carrying out the reaction at room temperature for 1.5 hours. After washing with 0.05% Tween-PBS, the wells were dried at 50°C, and Microscinti-0 (produced by Packard) was dispensed in 20 µl/well portions to measure the radioactivity of the ^{125}I-labeled human VEGF linked to each well using Top Count (produced by Packard).

The results are shown in Fig. 4. It was shown that soluble human VEGF receptors Flt-1 7N and Flt-1 3N inhibit binding of ^{125}I-labeled human VEGF to soluble human VEGF receptor Flt-1 7N in a concentration dependent manner. Since the soluble human VEGF receptors Flt-1 7N and Flt-1 3N showed similar degree of the human VEGF binding activity, it was revealed that the human VEGF binds to the Flt-1 3N moiety (the 1st to 338th positions from the N-terminal amino acid including signal sequence).

(8) Expression of human VEGF in insect cells

The human VEGF was obtained in the following manner. A 4×10^7 portion of High Five cells were suspended in 30 ml of EX-CELL™ 400 medium (produced by JRH Biosciences) contained in a 175 cm^2 flask (produced by Greiner) and allowed to stand at room temperature for 1 hour to effect adhesion of the cells to the flask. A 1 ml portion of a solution containing about 1 to 3×10^8 PFU/ml of human VEGF recombinant baculovirus particles obtained in accordance with the

known method (*Cell Growth & Differentiation*, 7, 213 (1996)) was added to the flask to carry out infection at room temperature for 2 hours. The culture supernatant was removed and 30 ml of fresh EX-CELL™ 400 medium was added to carry out 3 to 4 days of culturing at 27°C. After completion of the culturing, the cultur supernatant was collected and centrifuged at 1,500 x g for 10 minutes to obtain a supernatant fluid.

A column was packed with about 40 ml of heparin-Sepharose CL-6B gel (produced by Pharmacia Biotech AB) and washed with 400 ml of 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 ml/minute. After washing, 1,500 ml of the culture medium containing human VEGF prepared in the above-described manner was passed through the heparin-Sepharose CL-6B column at a flow rate of 0.5 ml/minute. After washing with 400 ml of 20 mM Tris-HCl (pH 7.5) buffer at a flow rate of 0.5 ml/minute, 120 ml of each of 20 mM Tris-HCl (pH 7.5) buffers containing 0.2 M, 0.5 M and 1 M NaCl was passed through the column in that order to carry out stepwise elution of the proteins adsorbed to the heparin-Sepharose, and the eluate was fractionated in 8 ml portions. Proteins contained in each fraction were analyzed by SDS polyacrylamide gel electrophoresis, and 120 ml of fractions (0.5 to 1 M NaCl fractions) containing human VEGF were collected. After concentration using CentriPrep-10 (produced by Amicon), human VEGF was obtained as 4 ml of solution (protein concentration, 1.2 mg/ml).

2. Immunization of animals and preparation of antibody producing cells

A 50 µg portion of each of the antigens obtained in the above-described step 1-(4) was administered, together with 2 mg of aluminum hydroxide gel and 1×10^9 cells of pertussis vaccine (produced by Chiba Serum Institute), into 5-week-old female BALB/c mice (SLC Japan), B6C3F1 mice (Charles River Japan) or female SD rats (SLC Japan), and, starting on 2 weeks thereafter, 10 to 50 µg of the protein was administered once a week for a total of four times. Also, 1×10^7 of NIH3T3-Flt-1 cells were administered 6 times into three, 5 week old female BALB/c (SLC Japan) mice. Blood samples were collected from the fundus of the eye or the caudal vein, their serum antibody titers were examined by the enzyme immunoassay described in the following, and spleens were excised from mice or rats showing sufficient antibody titer 3 days after the final immunization. In this connection, immunization was not induced in the 5-week-old female BALB/c to which NIH3T3-Flt-1 cells were administered, so that the antibody titer upon soluble Flt-1 7N was not increased.

The thus excised spleen was cut to pieces in MEM medium (produced by Nissui Pharmaceutical), unbound using a pair of forceps and then centrifuged (1,200 rpm for 5 minutes). The resulting supernatant was discarded, and the thus obtained sediment was treated with Tris-ammonium chloride buffer (pH 7.65) for 1 to 2 minutes to eliminate erythrocytes, washed three times with MEM medium and used in cell fusion.

3. Enzyme immunoassay

With regard to the measurement of antisera derived from mice or rats immunized with the soluble human Flt-1 7N and Flt-1 3N obtained in the above-described step 1-(4) and culture supernatants of hybridomas, the soluble human VEGF receptors Flt-1 7N and Flt-1 3N obtained from the insect cell culture supernatant of 1-(4) were used as antigens. A 1 to 10 µg/ml PBS-diluted solution of each of the soluble human VEGF receptors Flt-1 7N and Flt-1 3N and the heparin column adsorption fraction of High Five cell culture supernatant obtained in the above-described step 1-(6) as a control antigen was dispensed in 50 µl/well portions into a 96 well plate for EIA (produced by Greiner) and allowed to stand overnight at 4°C for coating. After washing, PBS containing 1% bovine serum albumin (BSA) was dispensed in 100 µl/well portions and 1 hour of the reaction was carried out at room temperature to effect blocking of the remained active groups. After discarding 1% BSA-PBS, antiserum of immunized mouse or immunized rat and culture supernatant of a hybridoma were dispensed in 50 µl/well portions to carry out the reaction for 2 hours. After washing with 0.05% Tween-PBS, peroxidase-labeled rabbit anti-mouse immunoglobulin or peroxidase-labeled rabbit anti-rat immunoglobulin (both produced by DAKO) was dispensed in 50 µl/well portions and 1 hour of the reaction was carried out at room temperature, the plate was washed with 0.05% Tween-PBS and then color development was caused using ABTS substrate solution (2,2-azinobis(3-ethylbenzothiazole-6-sulfonic acid) ammonium salt) to measure maximum absorbance at OD415 nm using E max (produced by Molecular Devices).

4. Preparation of mouse myeloma cells

8-Azaguanine-resistant mouse myeloma cell line P3U1 was cultured using normal medium to secure 2×10^7 or more of the cells for use in cell fusion as the parent cell line.

5. Preparation of hybridoma

The mouse spleen cells or rat spleen cells obtained in the above-described section 2 and the myeloma cells

obtained in the above section 4 were mixed to a ratio of 10:1 and centrifuged (1,200 rpm for 5 minutes), the supernatant was discarded, th precipitated cells were thoroughly loosened to which, while stirring at 37°C, were subsequently added a mixed solution of 2 g polyethylene glycol-1000 (PEG-1000), 2 ml MEM medium and 0.7 ml DMSO in an amount of 0.2 to 1 ml/10^8 mouse myeloma cells and then 1 to 2 ml of MEM medium several times at 1 to 2 minute intervals, and then the total volume was adjusted to 50 ml by adding MEM medium. After centrifugation (900 rpm for 5 minutes), the supernatant was discarded and the thus obtained cells were gently loosened and then gently suspended in 100 ml of HAT medium by repeated drawing up into and discharging from a graduated pipette.

The suspension was dispensed in 100 µl portions into wells of a 96 well culture plate and cultured at 37°C for 10 to 14 days in an atmosphere of 5% CO_2 in a 5% CO_2 incubator. The resulting culture supernatant was examined by the enzyme immunoassay method described in Example 1-3 to select wells which reacted specifically with the soluble human VEGF receptor Flt-1 7N or Flt-1 3N obtained in the above-described step 1-(4) but did not react with the control antigen obtained in the step 1-(6), and then cloning was repeated twice by changing the medium to HT medium and normal medium to establish hybridomas capable of producing anti-human VEGF receptor Flt-1 monoclonal antibodies. The results are shown in the following table.

Table 1

Animal	Head	Immunogen	Screening source	Wells screened	The number of hybridomas established
Balb/c mouse	3	NIH3T3-Flt-1	Flt 7N	-	-
SD rat	1	Flt 7N	Flt 7N	1008	3 (KM1733,1735,1736)
Balb/c mouse	1	Flt 7N	Flt 7N	672	5 (KM1737,1739,1740, 1742,1743)
SD rat	1	Flt 7N	Flt 7N	1176	3 (KM1745,1746,1747)
B3C3F1 mouse	1	Flt 7N	Flt 3N	672	3 (KM1748,1749,1750)
Balb/c mouse	1	Flt 7N	Flt 3N	420	3 (KM1730,1731,1732)

When hybridomas obtained from one Balb/c mouse and two SD rats immunized with the soluble human VEGF receptor Flt-1 7N prepared in the above-described step 1-(4) were screened for about 672 wells and about 2,184 wells, respectively, using the soluble human VEGF receptor Flt-1 7N, respective 5 clones and 6 clones of anti-human VEGF receptor Flt-1 monoclonal antibodies were obtained, and they were named KM1737, KM1739, KM1740, KM1742 and KM1743 and KM1733, KM1735, KM1736, KM1745, KM1746 and KM1747, respectively. None of these clones showed the action to inhibit binding of human VEGF to Flt-1 as shown in the following section 8. Additionally, KM1735, KM1736, KM1742, KM1743 and KM1745 reacted with human VEGF receptor Flt-1 expression cells by the immunocyte staining method described in the following section 10, but the reaction was extremely weak in comparison with KM1730, KM1731 and KM1732.

On the other hand, when hybridomas obtained from one B3C3F1 mouse and one Balb/c mouse immunized with the soluble human VEGF receptor Flt-1 7N prepared in the above-described step 1-(4) were screened for about 672 wells and about 420 wells, respectively, using the soluble human VEGF receptor Flt-1 3N, 3 clones for each of anti-human VEGF receptor Flt-1 monoclonal antibodies were obtained, and they were named KM1748, KM1749 and KM1750 and KM1730, KM1731 and KM1732, respectively. Of these clones, three clones KM1732, KM1748 and KM1750 showed the action to inhibit binding of human VEGF to Flt-1 as shown in the following section 8. Additionally, three clones KM1730, KM1731 and KM1732 reacted markedly strongly with human VEGF receptor Flt-1 expression cells by the immunocyte staining method described in the following section 10.

The antibody class of these monoclonal antibodies was determined by enzyme immunoassay using Subclass Typing Kit (produced by Zymed). The results are shown in the following table.

Table 2

Monoclonal antibody	Antibody subclass
KM1733	mouse IgG2a
KM1735	rat IgG1

Table 2 (continued)

M noclonal antibody	Antibody subclass
KM1736	rat IgG2a
KM1737	mous IgG1
KM1739	mouse IgG1
KM1740	mouse IgG1
KM1742	mouse IgG1
KM1743	mouse IgG1
KM1745	rat IgG2a
KM1746	rat IgG1
KM1747	rat IgG1
KM1748	mouse IgG2b
KM1749	mouse IgG1
KM1750	mouse IgG2b
KM1730	mouse IgG1
KM1731	mouse IgG2a
KM1732	mouse IgG1

All of the monoclonal antibodies established in the present invention were IgG class.

6. Purification of monoclonal antibody

The hybridomas obtained in the above section 5 were respectively administered to pristane-treated female nude mice (Balb/c) of 8 weeks of age by intraperitoneal injection at a dose of 5 to 20×10^6 cells per animal. The hybridomas caused ascites tumor formation in 10 to 21 days. The ascitic fluid was collected from each ascitic fluid-carrying mouse (1 to 8 ml per animal), centrifuged (3,000 rpm for 5 minutes) for removing solid matter and then purified by a caprylic acid precipitation method (*Antibodies - A Laboratory Manual*).

7. Confirmation of the specificity of monoclonal antibodies

Specificity of the anti-human VEGF receptor Flt-1 monoclonal antibodies described in the above-described section 5 was confirmed using the enzyme immunoassay method described in the above-described section 3.

The results are shown in Fig. 5. Among the monoclonal antibodies obtained by preparing hybridomas from mice and rats immunized with Flt-1 7N and selected using Flt-1 7N (KM1733, KM1735, KM1736, KM1737, KM1739, KM1740, KM1742 , KM1743, KM1745, KM1746 and KM1747), only KM1740 reacted with Flt-1 7N and Flt-1 3N, revealing that it recognizes an epitope which is present in a region of the 1st to 338th positions from the N-terminal amino acid of Flt-1 (including signal sequence). Since the remaining 10 clones reacted with Flt-1 7N but not with Flt-1 3N, it was revealed that they recognize an epitope which is present in a region of the 339th to 750th positions from the N-terminal amino acid of Flt-1 (including signal sequence). On the other hand, since all of the monoclonal antibodies obtained by preparing hybridomas from mice immunized with Flt-1 7N and selecting using Flt-1 3N (KM1748, KM1749, KM1750, KM1730, KM1731 and KM1732) reacted with Flt-1 7N and Flt-1 3N, it was revealed that they recognize an epitope which is present in a region of the 1st to 338th positions from the N-terminal amino acid of Flt-1 (including signal sequence).

8. Confirmation of the activity of anti-Flt-1 monoclonal antibodies to inhibit binding of a human VEGF to a human VEGF receptor Flt-1

The activity of the anti-human VEGF receptor Flt-1 monoclonal antibodies described in the above-described section 5 to inhibit binding of human VEGF to human VEGF receptor Flt-1 was confirmed in the following manner.

Methanol was dispensed in 100 µl portions into wells of a 96 well MultiScreen-IP Plate (produced by Millipore) to

give hydrophilic nature to the PVDF membrane on the bottom of th plate. After washing with water, the soluble human VEGF receptor Flt-1 7N diluted with PBS to a concentration of 1.6 μg/ml was dispensed in 50 μl/well portions and then allowed to stand overnight at 4°C to effect its adsorption. After washing, PBS containing 1% bovine serum albumin (BSA) is dispensed in 50 μl/well portions and 1 hour of the reaction was carried out at room temperature to effect blocking of the remained activ groups. After washing with PBS, each hybridoma culture supernatant or a purified monoclonal antibody diluted with 1% BSA-PBS containing 0.5 M NaCl (0.01 to 7.29 μg/ml) was dispensed in 50 μl/well portions and then 3 ng/ml of ^{125}I-labeled human VEGF (produced by Amersham) was dispensed in 50 μl/well portions, subsequently carrying out the reaction at room temperature for 1.5 hours. After washing with 0.05% Tween-PBS, the wells were dried at 50°C, and Microscinti-0 (produced by Packard) was dispensed in 30 μl/well portions to measure the radioactivity of the ^{125}I-labeled human VEGF linked to each well using Top Count (produced by Packard).

Results of the examination of activities of hybridoma culture supernatants are shown in Fig. 6. Among 17 established monoclonal antibodies, three monoclonal antibodies, KM1748, KM1750 and KM1732 inhibited binding of human VEGF to human VEGF receptor Flt-1 at inhibition ratios of 62.6%, 66.3% and 83.1%, respectively.

In general, screening of monoclonal antibody producing hybridomas is carried out using the same protein as the antigen used as the immunogen. A total of 11 monoclonal antibodies selected using Flt-1 7N as the immunogen showed no binding inhibition activity, and, among 6 monoclonal antibodies selected using Flt-1 3N (KM1748, KM1749, KM1750, KM1730, KM1731 and KM1732), KM1748, KM1750 and KM1732 showed the binding inhibition activity. It was an unexpected effect that monoclonal antibodies having the binding inhibition activity were obtained by the use of Flt-1 3N in the screening of hybridomas. Thus, it was revealed that Flt-1 3N is markedly important in establishing monoclonal antibodies having the binding inhibition activity.

Fig. 7 shows results of the examination of binding inhibition activity using purified anti-Flt-1 monoclonal antibodies KM1732, KM1748 and KM1750. These antibodies KM1732, KM1748 and KM1750 inhibited binding of human VEGF to human VEGF receptor Flt-1 in a concentration dependent manner. Concentrations of KM1732, KM1748 and KM1750, which indicate 50% inhibition of the binding of human VEGF to human VEGF receptor Flt-1 (IC_{50}), were 1.1, 1.3 and 2.0 μg/ml, respectively. On the other hand, an anti-sialyl-Le^a monoclonal antibody KM231 of a mouse IgG1 class (*Anticancer Research*, 10, 1579 (1990)) used as the control showed no inhibition activity.

9. Confirmation of the activity of anti-Flt-1 monoclonal antibodies to inhibit binding of human VEGF to human VEGF receptor Flt-1 expression cells

The activity of the anti-human VEGF receptor Flt-1 monoclonal antibodies KM1732, KM1748 and KM1750 to inhibit binding of human VEGF to human VEGF receptor Flt-1 was confirmed in the following manner.

PBS containing 1% bovine serum albumin (BSA) was dispensed in 100 μl portions into wells of a 96 well Multi-Screen-HV Plate (produced by Millipore), 1 hour of the reaction was carried out at room temperature to effect blocking of the active groups in the wells and then NIH3T3-Flt-1 cells suspended in 1% BSA-PBS containing 0.05% NaN_3 were dispensed in 5×10^4 cells/well portions. After washing with 1% BSA-PBS, a purified monoclonal antibody (0.01 to 7.29 μg/ml) was dispensed in 50 μl/well portions and then 3 ng/ml of ^{125}I-labeled human VEGF (produced by Amersham) was dispensed in 50 μl/well portions and the reaction was carried out under cooling for 2 hours. After washing with PBS, the wells were dried at 50°C, and Microscinti-0 (produced by Packard) was dispensed in 30 μl/well portions to measure the radioactivity of the ^{125}I-labeled human VEGF linked to each well using Top Count (produced by Packard).

Fig. 8 shows results of the examination of binding inhibition activity using purified anti-Flt-1 monoclonal antibodies KM1732, KM1748 and KM1750. These antibodies KM1732, KM1748 and KM1750 inhibited binding of human VEGF to NIH3T3-Flt-1 cells in a concentration dependent manner. Concentrations of KM1732, KM1748 and KM1750, which indicate 50% inhibition of the binding of human VEGF to NIH3T3-Flt-1 cells (IC_{50}), were 0.050, 0.037 and 0.041 μg/ml, respectively. On the other hand, the anti-sialyl-Le^a monoclonal antibody KM231 of a mouse IgG1 class used as the control showed no inhibition activity.

10. Confirmation of the reactivity of monoclonal antibodies with human VEGF receptor Flt-1 expression cells

Specificity of the anti-human VEGF receptor Flt-1 monoclonal antibodies described in the above-described section 5 was confirmed using immunocyte staining method in accordance the following procedure.

A total of 5×10^5 cells of each of human VEGF receptor Flt-1 expression NIH3T3 cells (NIH3T3-Flt-1) and control NIH3T3 cells (NIH3T3-Neo) (*Oncogene*, 10, 135 (1995)) were suspended in 100 μl of a buffer solution for immunocyte staining use (PBS containing 1% BSA, 0.02% EDTA and 0.05% sodium azide) and dispensed in a round bottom 96 well plate. After centrifugation at 4°C and at 350 x g for 1 minute, the supernatant fluid was discarded and the resulting cells were mixed with 50 μl of a hybridoma culture supernatant or purified antibody (10 μg/ml) and reaction was carried out at 4°C for 30 minutes. After the reaction, 200 μl of the buffer solution for immunocyte staining use was added to each well, and the cells were washed by centrifugation at 4°C and 350 x g for 1 minute followed by discarding the resulting

supernatant. After repeating this washing step twice, the cells were mixed with 50 µl of the buffer solution for immunocyte staining use containing 1 µg/ml of an FITC-labeled anti-mouse immunoglobulin antibody or FITC-labeled anti-rat immunoglobulin antibody (produced by Wako Pure Chemical Industries), and the reaction was carried out at 4°C for 30 minutes. After this reaction, the above-described washing step was repeated three times and then analysis was carried out using Flow Cytometer (produced by Coulter).

The results are shown in Fig. 9. The anti-human VEGF receptor Flt-1 monoclonal antibodies KM1730, KM1731 and KM1732 did not react with the control cells but specifically reacted in significant amounts with the Flt-1 expression cells. Neither, the anti-human VEGF receptor Flt-1 monoclonal antibody KM1748 (10 µg/ml) nor the hybridoma culture supernatant KM1748 reacted with the control cells. Each specifically reacted in significant amounts with the Flt-1 expression cells (B). As the results, it was discovered that the monoclonal antibodies KM1730, KM1731, KM1732, KM1748 and KM1750 specifically recognize the human VEGF receptor Flt-1 on the cell surface. On the other hand, KM1735, KM1736, KM1742, KM1743 and KM1745 only weakly reacted with the human VEGF receptor Flt-1 expression cells in comparison with KM1730, KM1731, KM1732, KM1748 and KM1750. 11. Detection of human VEGF receptor Flt-1 by Western blotting using monoclonal antibody

Cell membrane components were prepared from NIH3T3-Flt-1 cells and control NIH3T3 cells (NIH3T3-Neo) in accordance with a known method (*Cancer Research*, 46, 4438 (1986)) and subjected to electrophoresis by the SDS-PAGE method. The SDS-PAGE was carried out in accordance with a known method (*Anticancer Research*, 12, 1121 (1992)) by subjecting 15 µg, as protein per lane, of the cell membrane components to the electrophoresis using a 5 to 20% gradient gel (produced by Atto) under reducing conditions. The thus treated proteins were transferred to a PVDF membrane in accordance with a known method (*Anticancer Research*, 12, 1121, (1992)). Next, the PVDF membrane was allowed to react with PBS containing 1% BSA at room temperature for 30 minutes to effect blocking and then to react with the culture supernatant of the anti-human VEGF receptor Flt-1 monoclonal antibody KM1737 overnight at 4°C. The thus treated membrane was washed with PBS containing 0.05% Tween and then allowed to react with peroxidase-labeled goat anti-mouse IgG (5,000 times dilution: produced by Chemicon) at room temperature for 2 hours. After washing with 0.05% Tween-containing PBS, bands to which the anti-human VEGF receptor Flt-1 monoclonal antibody KM1737 was linked were detected using ECL™ Western blotting detection reagents (produced by Amersham).

The results are shown in Fig. 10. It was confirmed that the anti-human VEGF receptor Flt-1 monoclonal antibody KM1737 can detect the human VEGF receptor Flt-1 of 180 kilo dalton in molecular weight expressed in the NIH3T3-Flt-1 cells.

12. Detection of soluble human VEGF receptor Flt-1 using monoclonal antibody

The anti-human VEGF receptor Flt-1 monoclonal antibody KM1732 was diluted with PBS to a concentration of 10 µg/ml and dispensed in 50 µl/well portions into a 96 well plate for EIA (produced by Greiner) and allowed to stand overnight at 4°C for coating. After washing, PBS containing 1% bovine serum albumin (BSA) was dispensed in 100 µl/well portions and 1 hour of the reaction was carried out at room temperature to effect blocking of the remained active groups. After discarding 1% BSA-PBS, the purified soluble human VEGF receptors Flt-1 7N and Flt-1 3N obtained in the above-described step 1-(4) and diluted with 1% BSA-PBS to a concentration of 1,000 to 0.0056 ng/ml were allowed to react with the antibody overnight at 4°C. After washing with 0.05% Tween-PBS, the anti-human VEGF receptor Flt-1 monoclonal antibody KM1730 labeled with biotin by a known method (*Enzyme Antibody Method*: published by Gakusai Kikaku, 1985) was diluted with 1% BSA-PBS to a concentration of 0.1 µg/ml and dispensed in 50 µl/well portions to carry out the reaction at room temperature for 2 hours. After washing with 0.05% Tween-PBS, avidin-labeled peroxidase (produced by Vector) diluted 4,000 times with 1% BSA-PBS was dispensed in 50 µl/well portions to carry out the reaction at room temperature for 1 hour. After washing with 0.05% Tween-PBS, color development was caused using ABTS substrate solution (2,2-azinobis(3-ethylbenzothiazole-6-sulfonic acid) ammonium salt) to measure absorbance at OD415 nm using E max (produced by Molecular Devices).

The results are shown in Fig. 11. As the results, it was found that the soluble human VEGF receptors Flt-1 3N and Flt-1 7N can be measured from minimum concentrations of 0.46 ng/ml and 1.37 ng/ml, respectively, by the use of the anti-human VEGF receptor Flt-1 monoclonal antibody KM1732 and the biotin-labeled anti-human VEGF receptor Flt-1 monoclonal antibody KM1730.

13. Confirmation of the reactivity of monoclonal antibodies with human vascular endothelial cells HUVEC

The reactivity of anti-human VEGF receptor Flt-1 monoclonal antibodies described in the above-described section 5 with human vascular endothelial cells HUVEC was confirmed by immunocyte staining in the following manner.

A total of 2×10^5 cells of human umbilical vein endothelial cells (HUVEC) were suspended in 100 µl of a buffer solution for immunocyte staining use (PBS containing 1% BSA, 0.02% EDTA and 0.05% sodium azide) and dispensed in a round bottom 96 well plate. After centrifugation at 4°C and at 350 x g for 1 minute, the supernatant fluid was discarded

and the resulting cells were mixed with 50 μl (10 μg/ml) of each of biotinated purified antibodies KM1730 and KM1750 and control antibodies thereof, individually, and subsequently incubated at 4°C for 30 minutes. As the control antibody of KM1730, an anti-MxA monoclonal antibody KM1135 (WO 96/05230) of IgG1 type which is the same subclass of KM1730 was used. As the control antibody of KM1750, an anti-T cell receptor γ chain monoclonal antibody KM365 (Japanese Published Unexamined Patent Application No. 491/90) of IgG2b which is the same subclass as KM1750 was used. Thereafter, 200 μl of the buffer solution for immunocyte staining use was added to each well, and the cells wer washed by carrying out centrifugation at 4°C and at 350 × g for 1 minute and then the resulting supernatant was discarded. After again repeating this washing step twice, the cells were mixed with 20 μl of the buffer solution for immunocyte staining use containing 5 μg/ml in concentration of Avidin-PE (Streptoavidin-R-Phycoerythrin) (produced by Gibco), and the reaction was carried out at 4°C for 30 minutes. After the reaction, the above-described washing step was repeated three times and then the analysis was carried out using Flow Cytometer (produced by Coulter).

The results are shown in Fig. 12. The anti-human VEGF receptor Flt-1 monoclonal antibodies KM1730 and KM1750 reacted with HUVEC when compared with their control antibodies. These results demonstrate that the monoclonal antibodies KM1730 and KM1750 can detect human VEGF receptor Flt-1 on human vascular endothelial cells.

14. Increase of the expression quantity of Flt-1 on HUVEC by VEGF stimulation

As a model of vascular endothelial cells in an angiogenesis region, changes in the expression of human VEGF receptor Flt-1 before and after stimulation with VEGF were examined using the anti-human VEGF receptor Flt-1 monoclonal antibody KM1730 in accordance with the following procedure.

A total of 4 to 6Ç10^5 cells of each of four lots of HUVEC (lot #4031, #4102, #2477 and #4723; purchased from Clonetics) were suspended in 20 ml of a medium (produced by KURABO) (control medium) composed of E-BM medium further supplemented with 5% fetal bovine serum (FBS), 10 ng/ml of human recombinant type epidermal growth factor (hEGF), 1 mg/ml of hydrocortisone, 50 mg/ml of gentamicin and 50 ng/ml of amphotericin, and the suspension was further mixed with 1.2 mg/ml of bovine brain extract (BBE) (produced by KURABO) as a growth factor and subjected to 2 to 3 days of culturing at 37¯ C. When the cells were proliferated into 1 to 2Ç10^6 cells, the medium was removed and replaced with 20 ml of fresh control medium to carry out a total of 2 days of culturing. After the culturing for 1 day, human VEGF was added to a final concentration of 5 ng/ml, and the cells after the additional culturing for 1 day were used as VEGF-stimulated cells. Cells cultured for 2 days without adding VEGF were used as control cells (VEGF-non-stimulated cells). After the culturing, the cells were collected to examine reactivity of the anti-human VEGF receptor Flt-1 monoclonal antibody KM1730 by the immunocyte staining method in accordance with the procedure described in the above section 13.

Results of the examination of its reactivity with the lot #2477 HUVEC are shown in Fig. 13. KM1730 reacted with VEGF-non-stimulated HUVEC but more strongly with VEGF-stimulated HUVEC. Reactivity of the control antibody KM1135 did not change independent of the VEGF stimulation or non-stimulation. Fig. 14 shows changes in the Flt-1 expression in four lots of HUVEC (lot #4031, #4102, #2477 and #4723) by VEGF stimulation. The expression quantity of Flt-1 which can express the reactivity of KM1730 as an index is shown as a relative value when reactivity of the control antibody is defined as 1. It was revealed that all of the four lots of HUVEC can express Flt-1 by VEGF non-stimulation, and the expression quantity of Flt-1 increases by the VEGF stimulation.

The increase of the expression quantity of Flt-1 and the reactivity of anti-Flt-1 monoclonal antibody in the VEGF-stimulated human vascular endothelial cells HUVEC as a model of angiogenesis shows that the monoclonal antibody is useful for the diagnosis or treatment of diseases in which their morbid states progress by the acceleration of angiogenesis caused by VEGF, such as tumors, rheumatoid arthritis, diabetic retinopathy, and the like.

INDUSTRIAL APPLICABILITY

The present invention renders possible provision of monoclonal antibodies that specifically binds to human VEGF receptor Flt-1 which is considered to be expressed specifically in vascular endothelial cells of human angiogenesis regions. The monoclonal antibodies of the present invention are useful for the immunological detection of human angiogenesis regions by immunocyte staining and for the diagnosis or treatment, through the inhibition of the biological activities of human VEGF, of diseases in which their morbid states progress by abnormal angiogenesis, such as proliferation or metastasis of solid tumors, arthritis in rheumatoid arthritis, diabetic retinopathy, retinopathy of prematurity, psoriasis, and the like.

SEQUENCE LISTING

SEQ ID NO:1
Sequence Length: 23
Sequence Type: nucleic acid
Strandedness: single
Topology: linear
Molecular Type: other nucleic acid, synthetic DNA
Sequence:
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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: ANTI-HUMAN VEGF RECEPTOR Flt-1 MONOCLONAL ANTIBODY

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: EP 97913427.7 (PCT/JP97/04259)
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(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: M/39246

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(B) TELEFAX: (089) 987304

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

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(2) INFORMATION FOR SEQ ID NO:2:

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Claims

1. A monoclonal antibody which specifically reacts with human VEGF receptor Flt-1.

2. The monoclonal antibody according to claim 1, which recognizes an epitope present in a region of the 1st to 750th positions from the N-terminal amino acid (including a signal sequence) of human VEGF receptor Flt-1.

3. The monoclonal antibody according to claim 2, which recognizes an epitope present in a region of the 1st to 338th positions from the N-terminal amino acid (including a signal sequence) of human VEGF receptor Flt-1.

4. The monoclonal antibody according to any one of claims 1 to 3, which specifically reacts with human VEGF receptor Flt-1 by immunocyte staining.

5. The monoclonal antibody according to any one of claims 1 to 3, which inhibits binding of human VEGF to human VEGF receptor Flt-1 and inhibits biological activities of human VEGF.

6. The monoclonal antibody according to claim 4, which is monoclonal antibody KM1730 belonging to mouse IgG1 subclass.

7. The monoclonal antibody according to claim 4, which is monoclonal antibody KM1731 belonging to mouse IgG2a subclass.

8. The monoclonal antibody according to claim 4 or 5, which is monoclonal antibody KM1732 belonging to mouse IgG1 subclass.

9. The monoclonal antibody according to claim 4 or 5, which is monoclonal antibody KM1748 belonging to mouse IgG2b subclass.

10. The monoclonal antibody according to claim 4 or 5, which is monoclonal antibody KM1750 belonging to mouse IgG2b subclass.

11. Hybridoma KM1730 (FERM BP-5697) which produces the monoclonal antibody of claim 6.

12. Hybridoma KM1731 (FERM BP-5718) which produces the monoclonal antibody of claim 7.

13. Hybridoma KM1732 (FERM BP-5698) which produces the monoclonal antibody of claim 8.

14. Hybridoma KM1748 (FERM BP-5699) which produces the monoclonal antibody of claim 9.

15. Hybridoma KM1750 (FERM BP-5700) which produces the monoclonal antibody of claim 10.

16. A method for immunologically detecting human VEGF receptor Flt-1, which comprises using the monoclonal antibody of any one of claims 1 to 10.

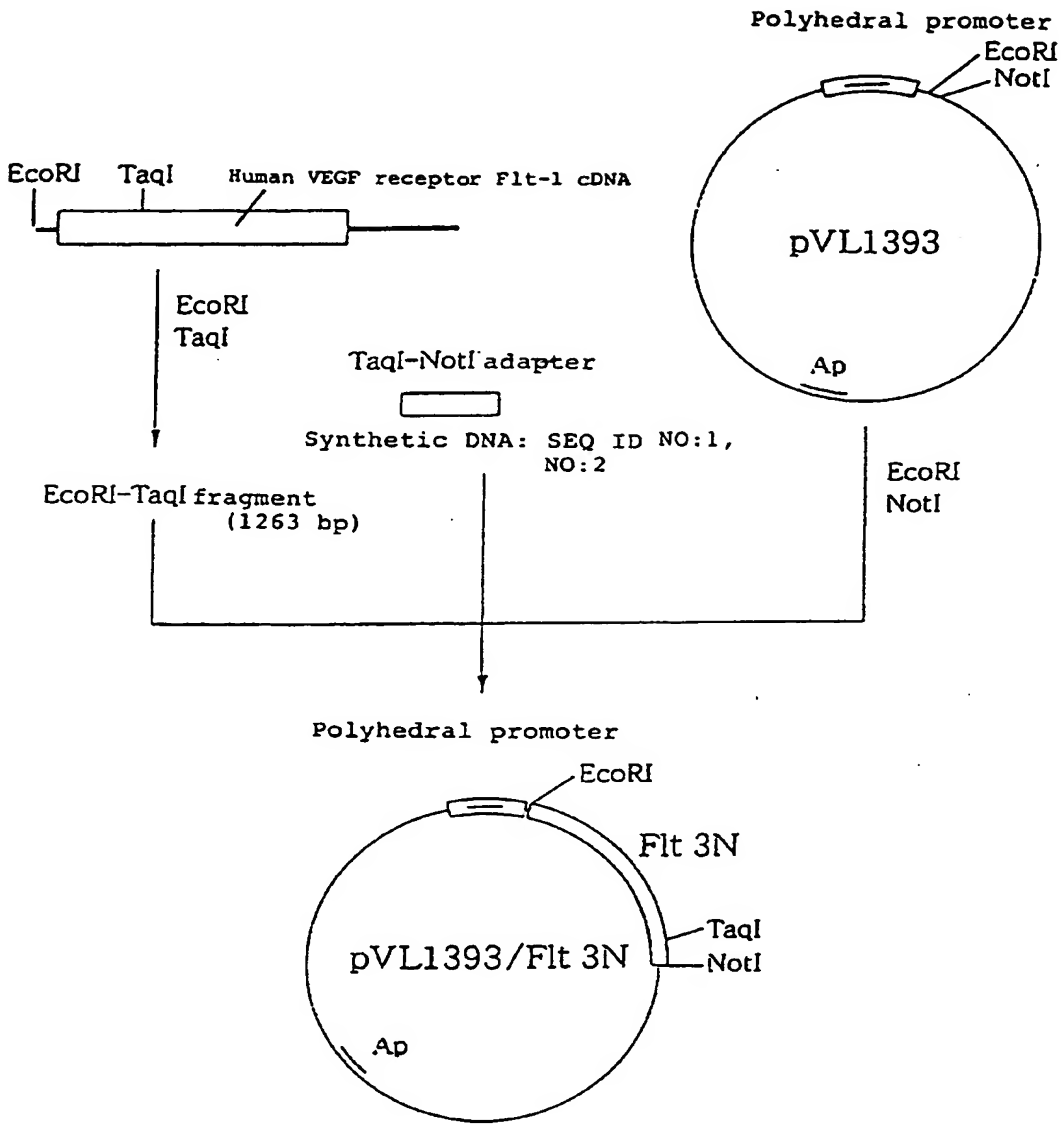
17. A method for immunologically detecting cells in which human VEGF receptor Flt-1 is expressed on the surface thereof, which comprises using the monoclonal antibody of any one of claims 1 to 10.

18. A method for immunologically detecting and determining soluble human VEGF receptor Flt-1, which comprises using the monoclonal antibody of any one of claims 1 to 10.

19. A method for inhibiting binding of human VEGF to human VEGF receptor Flt-1, which comprises using the monoclonal antibody of any one of claims 1 to 5 and 8 to 10.

20. A method for inhibiting biological activities of human VEGF, which comprises using the monoclonal antibody of any one of claims 1 to 5 and 8 to 10.

Fig. 1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NotI
EcoRI
TaqI
Human VEGF receptor Flt-1 cDNA
pVL1393
EcoRI
TaqI
TaqI-NotI adapter
Ap
Synthetic DNA: SEQ ID NO:1, NO:2
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TaqI
NotI
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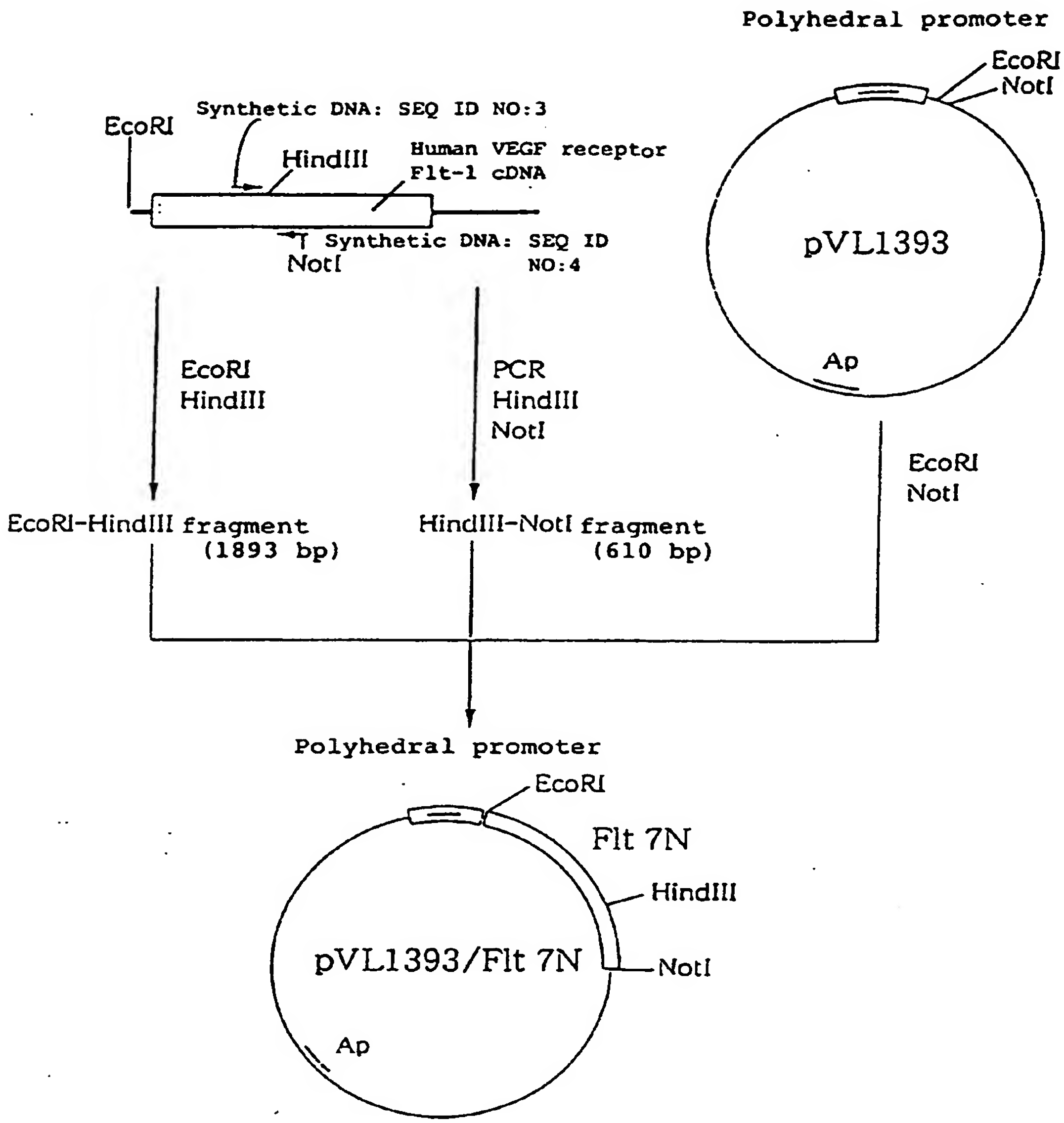
Ap

Fig. 2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Human VEGF receptor
Flt-1 cDNA
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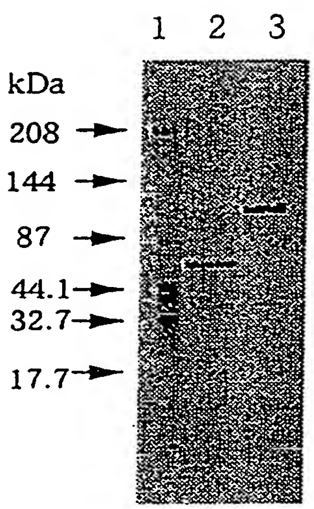
pVL1393
Ap
EcoRI
HindIII
PCR
HindIII
NotI
EcoRI
NotI
EcoRI-HindIII fragment (1893 bp)
HindIII-NotI fragment (610 bp)
Polyhedral promoter
EcoRI
Flt 7N
HindIII
NotI
pVL1393/Flt 7N
Ap

Fig. 3



Lane

1. Molecular weight marker
2. Flt-1 (3N)
3. Flt-1 (7N)

Fig. 4

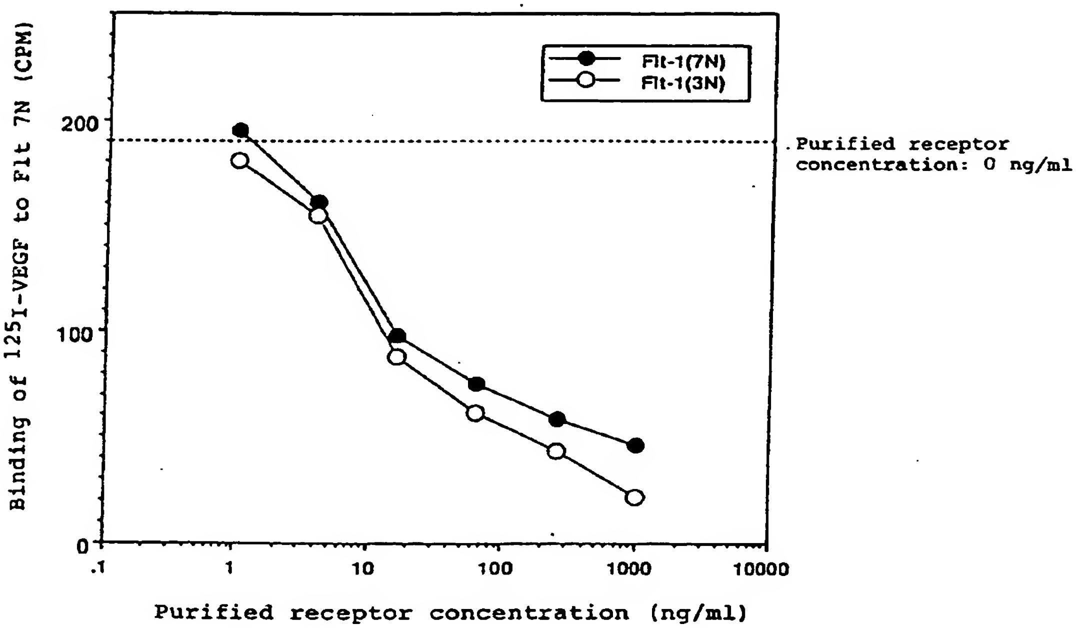

Flt-1(7N)
Flt-1(3N)
Purified receptor concentration: 0 ng/ml
Binding of 125I-VEGF to Flt 7N (CPM)
0
100
200
.1
1
10
100
1000
10000
Purified receptor concentration (ng/ml)

Fig. 5

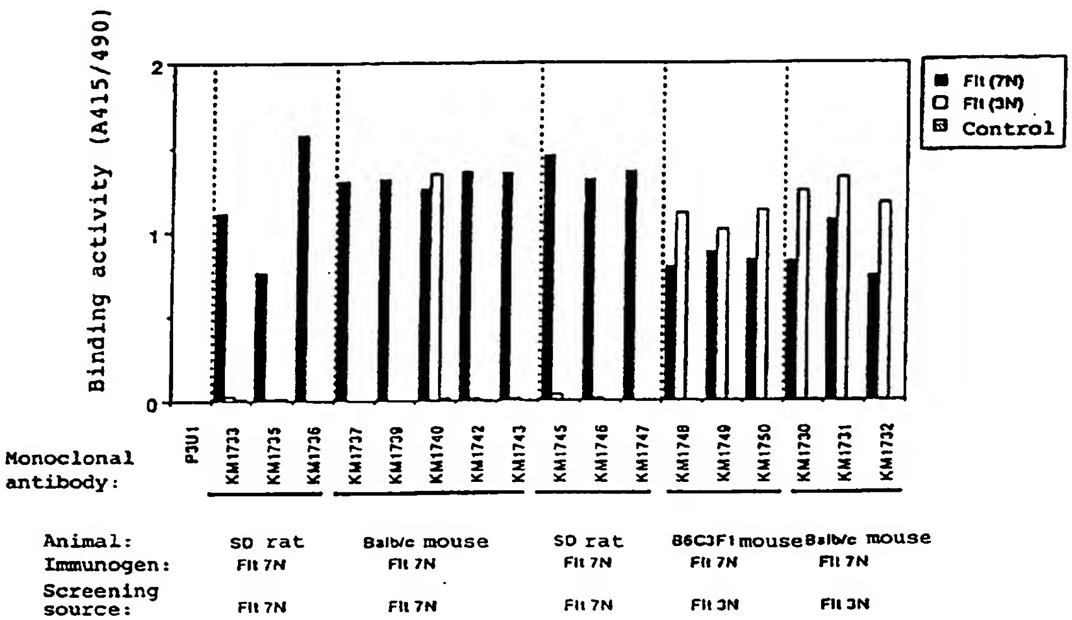

Binding activity (A415/490)
2
1
0
Flt (7N)
Flt (3N)
Control
Monoclonal antibody:
P3U1
KM1733
KM1735
KM1736
KM1737
KM1739
KM1740
KM1742
KM1743
KM1745
KM1746
KM1747
KM1748
KM1749
KM1750
KM1730
KM1731
KM1732
Animal:
SD rat
Balb/c mouse
SD rat
B6C3F1 mouse
Balb/c mouse
Immunogen:
Flt 7N
Flt 7N
Flt 7N
Flt 7N
Flt 7N
Screening source:
Flt 7N
Flt 7N
Flt 7N
Flt 3N
Flt 3N

Fig. 6

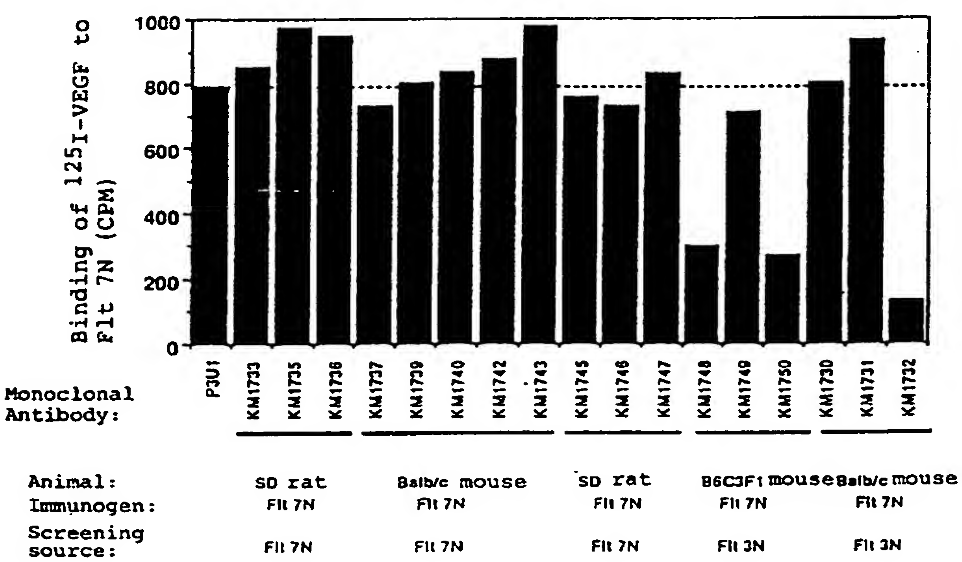

Binding of 125I-VEGF to Flt 7N (CPM)
1000
800
600
400
200
0
Monoclonal Antibody:
P3U1
KM1733
KM1735
KM1736
KM1737
KM1739
KM1740
KM1742
KM1743
KM1745
KM1746
KM1747
KM1748
KM1749
KM1750
KM1730
KM1731
KM1732
Animal:
SD rat
Balb/c mouse
SD rat
B6C3F1 mouse
Balb/c mouse
Immunogen:
Flt 7N
Flt 7N
Flt 7N
Flt 7N
Flt 7N
Screening source:
Flt 7N
Flt 7N
Flt 7N
Flt 3N
Flt 3N

Fig. 7

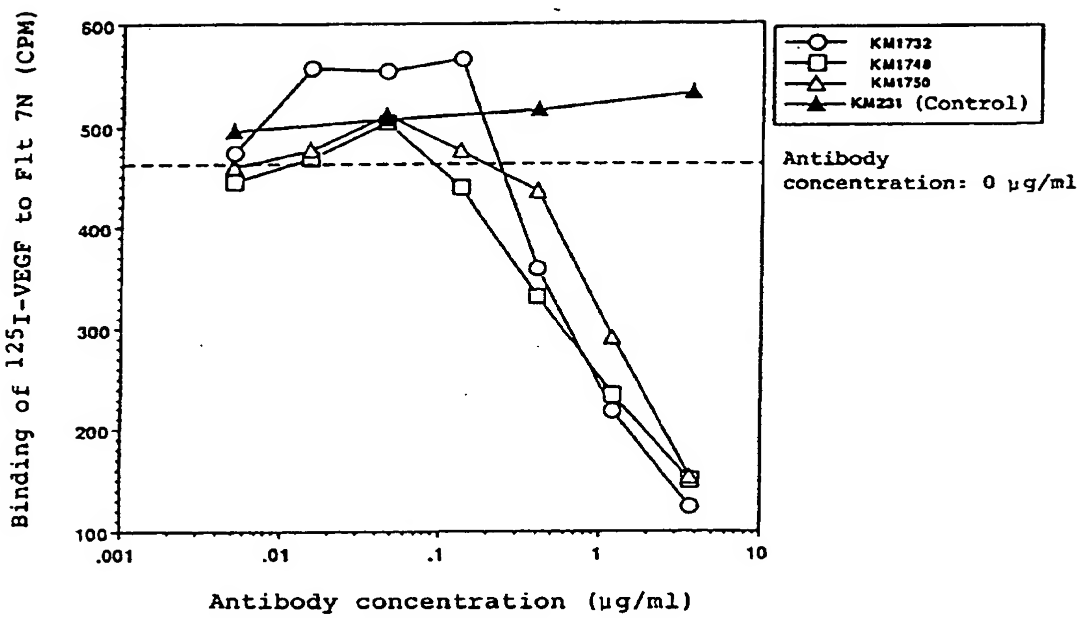

600
500
400
300
200
100
.001
.01
.1
1
10
Binding of 125I-VEGF to Flt 7N (CPM)
Antibody concentration (µg/ml)
KM1732
KM1748
KM1750
KM231 (Control)
Antibody concentration: 0 µg/ml

Fig. 8

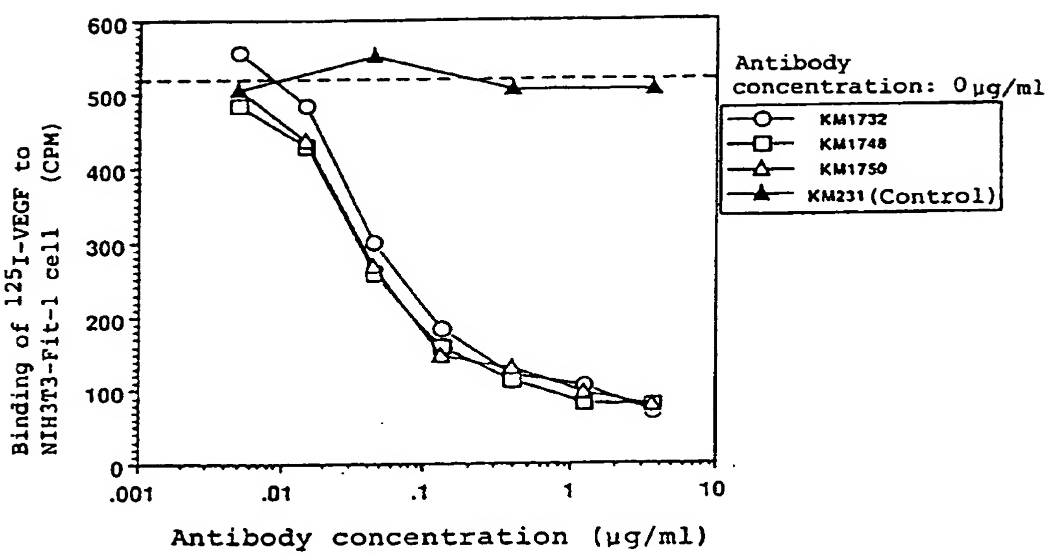

Binding of 125I-VEGF to NIH3T3-Flt-1 cell (CPM)
600
500
400
300
200
100
0
.001
.01
.1
1
10
Antibody concentration (μg/ml)
Antibody concentration: 0 μg/ml
KM1732
KM1748
KM1750
KM231 (Control)

Fig. 9

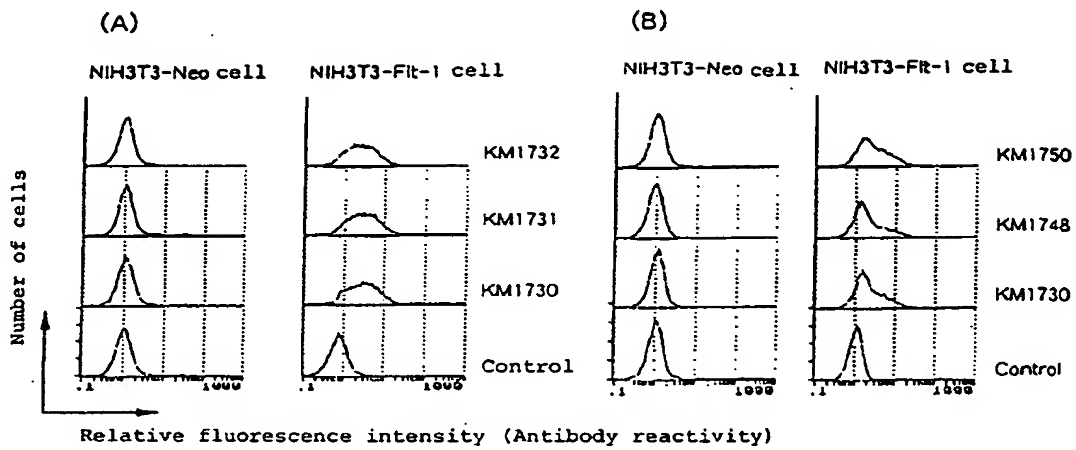

(A)
(B)
NIH3T3-Neo cell
NIH3T3-Flt-1 cell
NIH3T3-Neo cell
NIH3T3-Flt-1 cell
KM1732
KM1731
KM1730
Control
KM1750
KM1748
KM1730
Control
Number of cells
Relative fluorescence intensity (Antibody reactivity)

Fig. 10

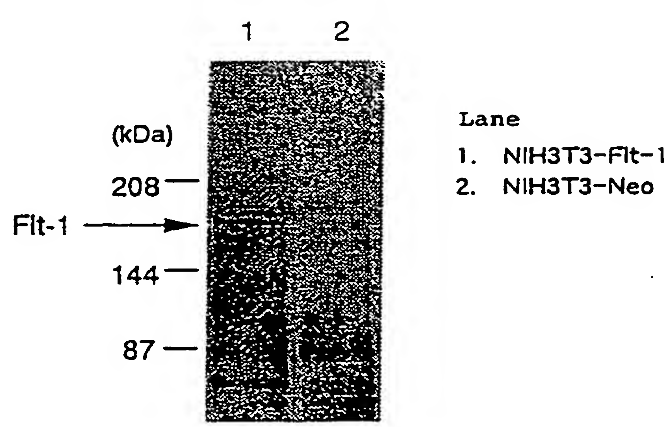
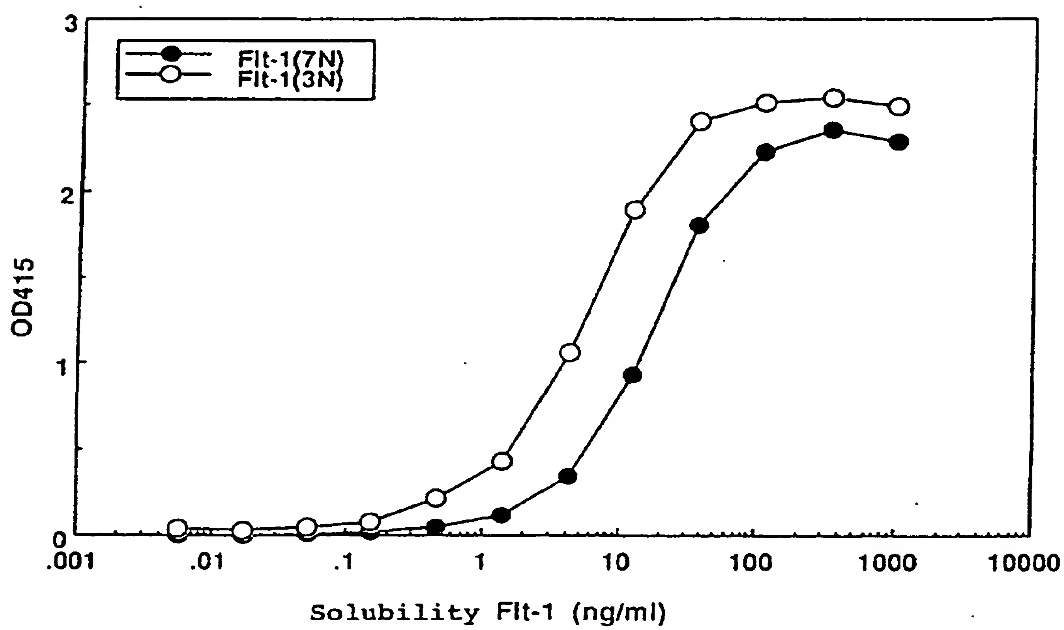

1
2
(kDa)
208
144
87
Flt-1
Lane
1. NIH3T3-Flt-1
2. NIH3T3-Neo

Fig. 11



Flt-1(7N)
Flt-1(3N)
3
2
1
0
OD415
.001
.01
.1
1
10
100
1000
10000
Solubility Flt-1 (ng/ml)

Fig. 12

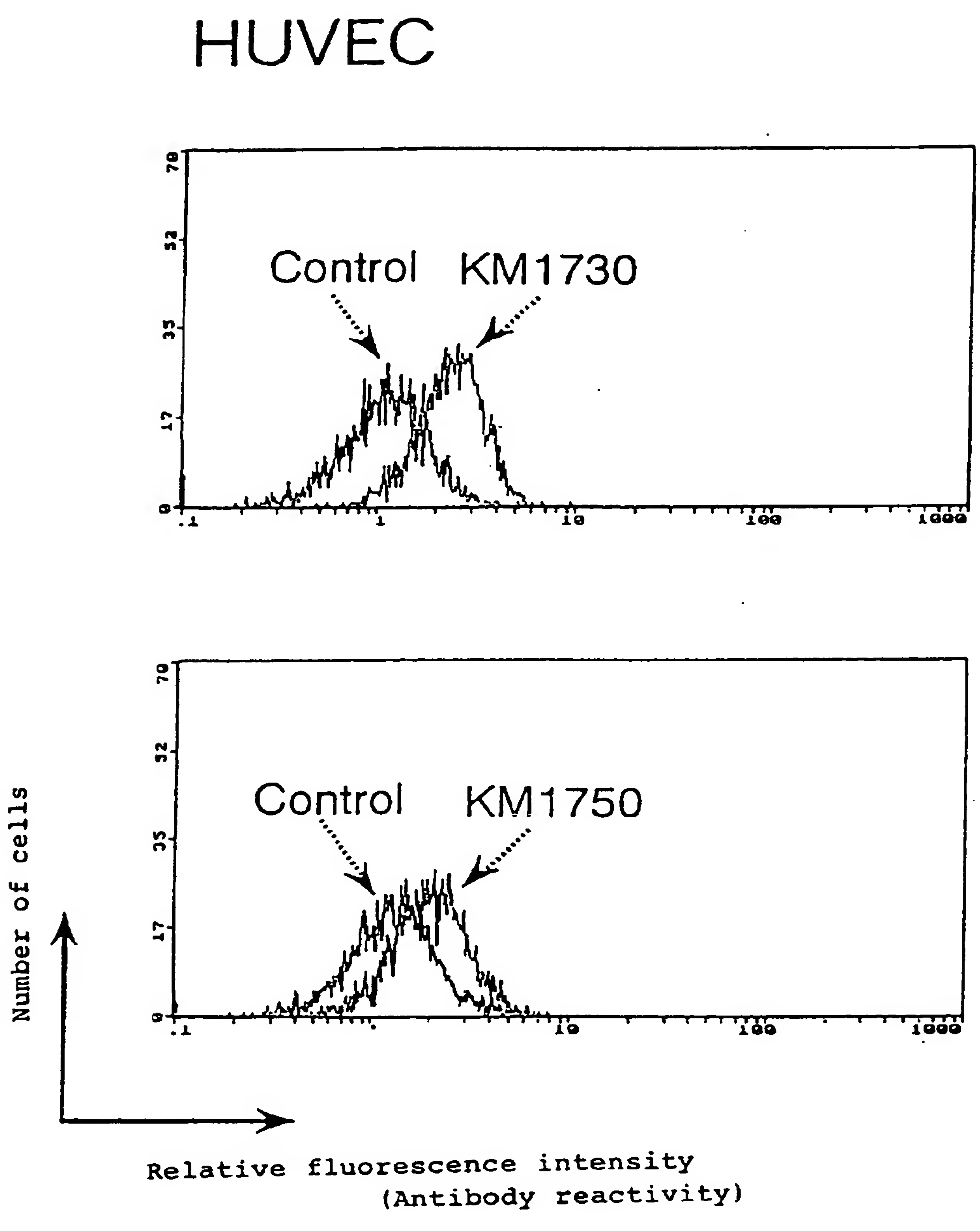

HUVEC
Control
KM1730
Control
KM1750
Number of cells
Relative fluorescence intensity
(Antibody reactivity)

Fig. 13

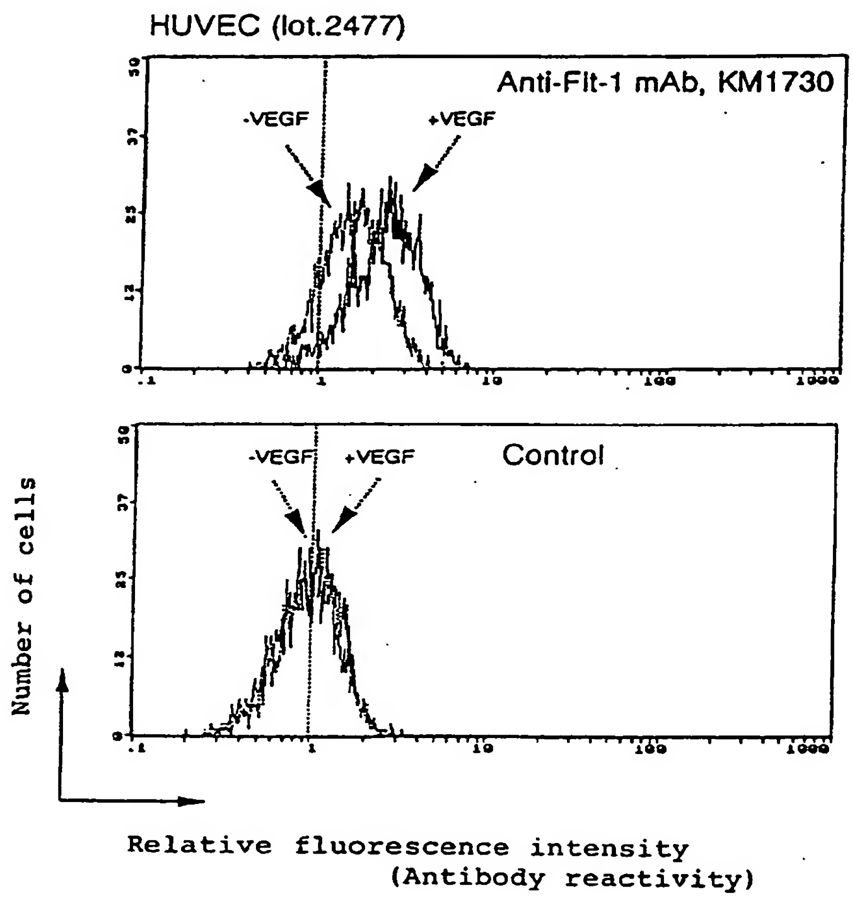
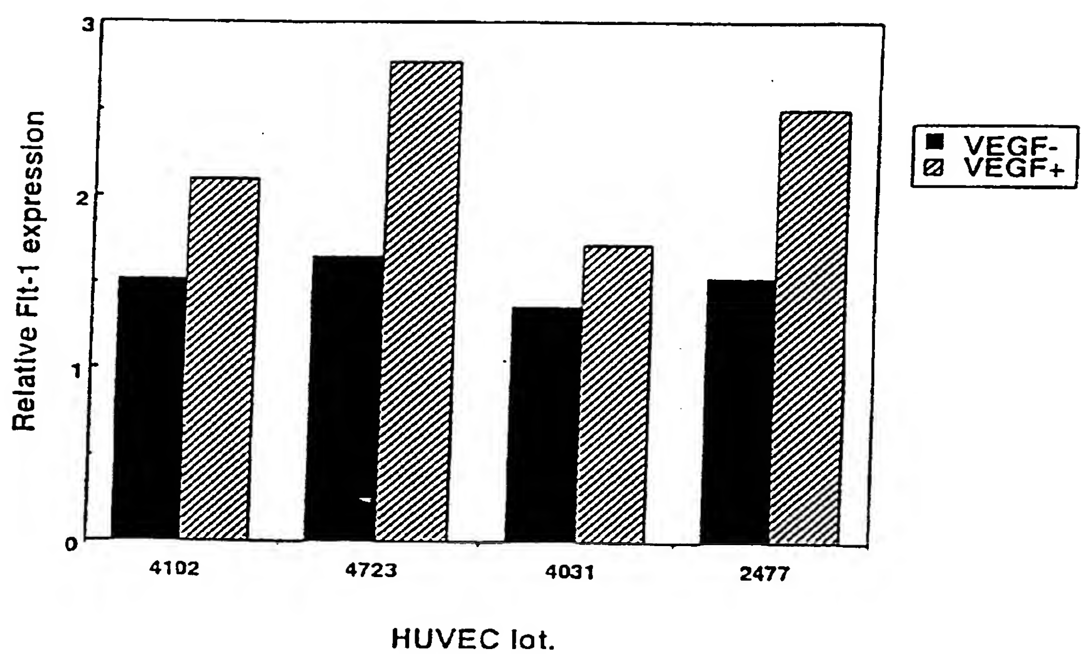

HUVEC (lot.2477)
Anti-Flt-1 mAb, KM1730
-VEGF
+VEGF
Control
-VEGF
+VEGF
Number of cells
Relative fluorescence intensity
(Antibody reactivity)

Fig. 14


Relative Flt-1 expression
3
2
1
0
VEGF-
VEGF+
4102
4723
4031
2477
HUVEC lot.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP97/04259

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl[6] C12P21/02, C12N15/06, C12N5/16, C07K16/28, A61K39/395, G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl[6] C12P21/02, C12N15/06, C12N5/16, C07K16/28, A61K39/395, G01N33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Oncogene, Vol. 5, (1990) Masabumi Shibuya et al. "Nucleotide Sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms famiy" p. 519-524	1 - 20
Y	Oncogene, Vol. 10, (1995) L. Seetharam et al. "A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF" p. 135-147	1 - 20
Y	Nature, Vol. 362, (1993) K. Jin Kim et al. "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo" p. 841-844	5, 20
Y	Science, Vol. 255, (1992) Carlie de Vries et al. "The fms-Like Tyrosine Kinase, a Receptor for Vascular Endothelial Growth Factor" p. 989-991	5, 20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
February 12, 1998 (12. 02. 98)	February 24, 1998 (24. 02. 98)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP97/04259

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Edited by Tamie Ando et al. "Manual for Experiments on Monoclonal Antibody (in Japanese)" (Kodansha Scientific) 1991, p. 8-84	1 - 20
Y	Cancer Res., Vol. 56, (1996) O. Melnyk et al. "Vascular Endothelial Growth Factor Promotes Tumor Dissemination by a Mechanism Distinct from Its Effect on Primary Tumor Growth" p. 921-924	5, 20

Form PCT/ISA/210 (continuation of second sheet) (July 1992)